DEFINITION OF ANTIGENIC DETERMINANTS IN DRUG HYPERSENSITIVE PATIENTS: AN INTEGRATED CLINICAL, CHEMICAL AND CELLULAR APPROACH TO QUANTIFY AND CHARACTERIZE THE DRUG SIGNALS PRESENTED TO T-LYMPHOCYTES

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

Monday Ogaba Ogese

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To George Edge (A friend indeed)

Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree

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Contents

Abbreviations

A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
ACN	Acetonitrile
ADR	Adverse drug reaction
ALP	Alkaline phosphatase
APC	Antigen presenting cells
CCR	Chemokine receptor (C-C) motif
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
cpm	Counts per minute
CRM	Chemically reactive metabolite
CSA	Cyclosporin
C_t	Cycle threshold
CTL	Cytotoxic T lymphocyte
CYP	Cytochrome P450 enzyme
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DHR	Drug hypersensitivity reaction
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
DNA	Dioxyribonucleic acid
DNCB	Dinitrochlorobenzene
DRESS	Drug reaction with eosinophilia and systemic symptoms
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked immunospot
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum

FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Reduced glutathione
HBSS	Hanks balanced salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN-γ	Interferon-gamma
HPLC	High-performance liquid chromatography
IgE	Immunoglobulin E
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motifs
LAT cells	Transmembrane adapter protein linker for the activation of T-
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MHC	Major Histocompatibility complex
Mins	Minutes
Mo-DC	Monocyte-derived dendritic cells
MRM	Multiple reaction monitoring
NHS	National health service
NK	Natural killer
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
pH	Power of hydrogen

pi	Pharmacological interaction
pKa	Acid dissociation constant
PKC	Protein kinase C
PPV	Positive predictive value
RNA	Ribonucleic acid
RIPA	Radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute
R_t	Retention time
SFC	Spot forming cell
SI	Stimulation index
SJS	Stevens-Johnson syndrome
SLP-76	SH_2 domain-containing leukocyte phospho-protein of 76kDa
SMX	Sulfamethoxazole
SMX.NOH	Sulfamethoxazole hydroxylamine
SMX.NO	Nitroso sulfamethoxazole
STAT	Signal Transducer and Activator of Transcription
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TEN	Toxic epidermal necrolysis
Th1	Type 1 helper cell
Th2	Type 2 helper cell
TNF-α	Tumour necrosis factor-α
Tregs	Regulatory T cells
TT	Tetanus toxoid
UK	United Kingdom
w/v	weight/volume
WHO	World Health Organisation
ZAP-70	ζ-chain-associated protein kinase

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Published papers

Kiorpelidou E, Foster B, Farrell J, **Ogese MO,** Faulkner L, Goldring CE, Park BK, Naisbitt DJ. IL-8 release from human neutrophils cultured with pro-haptenic chemical sensitizers. Chem Res Toxicol. 2012 Oct 15; 25(10):2054-6. doi: 10.1021/tx300350s. Epub 2012 Sep 12

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Gibson A, **Ogese MO**, Sullivan A, Wang E, Saide K, Whitaker P, Peckham D, Faulkner L, Park BK, Naisbitt DJ. Negative Regulation by PD-L1 during Drug-Specific Priming of IL-22-Secreting T Cells and the Influence of PD-1 on Effector T Cell Function. J Immunol. 2014 Mar 15;192(6):2611-21. doi: 10.4049/jimmunol.1302720. Epub 2014 Feb 7

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Submitted manuscript

M.O. Ogese, K. Saide, L. Faulkner, P. Whitaker, D. Peckham, A. Alfirevic, M. Pirmohamed, B. K. Park and D.J. Naisbitt. HLA-DQ allele restricted activation of nitroso-sulfamethoxazole-specific CD4 positive T-lymphocytes from patients with cystic fibrosis (submitted). Journal of clinical and experimental allergy (April, 2014).

Manuscripts in preparation

Monday O. Ogese, Rosalind E. Jenkins, James L. Maggs, Paul Whitaker, Daniel Peckham, Lee Faulkner, B. Kevin Park, and Dean J. Naisbitt. Characterization of peroxidases expressed in human antigen presenting cells and analysis of nitroso-sulphamethoxazole-myeloperoxidase binding (Chem Res Toxicol.).

Abstract

Idiosyncratic drug hypersensitivity remains a major challenge as it causes high morbidity and mortality. This is complicated by the multiple risk factors implicated and the inability to predict these reactions during the early stages of drug development. Thus, this study attempted to delineate the molecular pathomechanism(s) involved in sulfamethoxazole (SMX) hypersensitivity.

The reactive metabolite, nitroso-SMX (SMX.NO) generated through the hepatic bioactivation of SMX has long been hypothesised as a major trigger of these reactions. SMX hypersensitivity has been used as a paradigm to study the role of drug metabolism in the activation of T-cells as the synthetic nitroso metabolite is available for functional studies. Metabolism of SMX in hepatic tissue has been extensively studied. CYP2C9 and Myeloperoxidase (MPO) are implicated in the formation of SMX.NO. However, it is unclear whether the SMX.NO generated in the liver migrates to the skin; the primary target in SMX hypersensitivity. It is possible that localised SMX metabolism by immune cells resident in the skin are implicated in the observed reactions. ELISA data revealed SMX metabolism in EBV-transformed B-cells used as antigen presenting cells (APCs). SMX-metabolism was significantly inhibited by methimazole. Furthermore, Western blotting and RT-PCR analyses suggested the presence of low concentrations of MPO in EBV-transformed B-cells. Interestingly, RT-PCR revealed mRNA expression of flavine containing monooxygenases (FMO1-5), TPO and LPO but the protein levels of these enzymes were not detected in immune cells. Subsequent experiments involved the generation and LC-MS/MS characterization of SMX.NO-modified MPO adducts. Although SMX.NO formed both the sulphinamide and N-hydroxysulfinamide adducts, drug specific T-cell clones failed to proliferate in response to drug-modified peptides.

Since SMX.NO binds to multiple cellular proteins, it is assumed that peptides derived from the modified protein interact with a number of diverse HLA molecules to activate T-cells. However, the HLA molecules that interact with SMX.NO-modified peptides have not been defined. This study therefore examined the HLA molecules that present SMX.NO (derived peptides) to T-cells. T-cell clones (TCCs) were generated from 5 hypersensitive patients with cystic fibrosis. Fast growing TCCs from 2 SMX hypersensitive patients were used for HLA restriction studies. Drug-specific proliferative response, cytokine secretion and cytolytic markers were measured using [^{3}H]-thymidine incorporation and ELIspot assays. Anti-human class I and class II (DR, DP, and DQ) antibodies were used to determine HLA restriction of drug-specific T-cell activation. APCs expressing similar or different HLAs were used to define the alleles involved in the presentation of SMX.NO-derived antigens to T-cells. A total of 1578 clones were tested for SMX.NO reactivity. Seventy-seven $CD4^+$ clones were activated to proliferate and secrete IFN-ϒ, IL-5, IL-13 and granzyme-B by SMX.NO. Only one TCC was $CD8^+$No cross reactivity with SMX was observed. The SMX.NO-specific response of clones was blocked with antibodies against MHC class II and HLA-DQ. Clones from 2 patients (Patient 1: HLA-DQB1*05:01:01G/ DQB1*06:03:01G; Patient 2: HLA-DQB1*02:01:01G/DQB1*02:01:01G) were used to define the DQ alleles involved in the presentation of SMX.NO derived antigens. SMX.NO-specific responses were detected with heterologous APCs expressing HLA-DQB1*05:01 (patient 1) and HLA-DQB1*02:01 (patient 2), but not other HLA-DQB1 alleles.

Activation of PD-1 on T-cells is thought to inhibit antigen-specific T-cell priming and regulate T-cell differentiation. Thus, this study sought to measure the drug-specific activation of naïve T-cells after perturbation of PD-L1/PD-1 binding and investigate whether PD-1 signalling influences the differentiation of T-cells. Naive T-cells were co-cultured with monocyte-derived dendritic cells in the presence of SMX.NO for a period of 8 days (±PD-1/2 block) and T-cell priming investigated using readouts for proliferation and cytokine secretion. Priming of naïve T-cells against SMX.NO was found to be more effective when PD-L1 signalling was blocked. Drug-specific TCCs generated through priming *and* from hypersensitive patients were found to secrete IFN-γ, IL-5 and IL-13. More detailed analysis revealed two different cytokine signatures. Clones secreted either FasL/IL-22 or granzyme B. The FasL/IL22 secreting clones expressed the skin homing receptors CCR4, CCR10 and CLA and migrated in response to CCL17/CCL27. PD-1 was stably expressed at different levels on clones; however, PD-1 expression did not correlate with the strength of the antigen-specific proliferative response or the secretion of cytokines/cytolytic molecules.

In conclusion, this study used a variety of *in vitro* assays to investigate the multiple factors involved in the pathomechanism of SMX hypersensitivity. A clear understanding of mechanisms of drug hypersensitivity will provide insights that aid drug design and reduce the frequency of such reactions.

Chapter 1: General introduction

Contents

1.1 Adverse drug reactions (ADRs)

The use of drugs for prophylaxis, diagnosis, management and treatment of diseases is sometimes accompanied by ADRs, with 6-7% of all hospital admissions attributed to ADRs (Einarson 1993; Classen et al. 1997; Gomes and Demoly 2005). ADRs greatly impact the healthcare system and are a leading cause of mortality (Lazarou et al. 1998; Pirmohamed et al. 1998; Bharadwaj et al. 2012).They negatively affect the duration of hospital admissions and the cost of treatment (Bates et al. 1997; Classen et al. 1997); thus, ADRs are a major concern for the patients, health care providers and the pharmaceutical industry. Many drugs have either been withdrawn or their use restricted in the past due to ADRs (table 1.1). Scientists in both the industry and the academia have invested time and resources to understand the molecular mechanism of ADRs.

Pharmacovigilance is the aspect of clinical practice charged with the monitoring of ADRs. The manifestations and severity of ADRs are dependent on a number of susceptibility factors including gender, age, genetic predisposition, disease state and the chemical properties of the drug involved. Clinical presentation of ADRs are heterogeneous and may include skin rash, urticaria, itching, fixed dose eruptions, angioedema, Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (Sharma and Sethuraman 1996; Sharma et al. 2001). The skin is the most affected organ following the activation of the relatively latent immune system by drugs (Arndt and Jick 1976; Hunziker et al. 1997).

Table1.1–Safety outcomes of various drugs due to adverse reactions (adapted from Talbot, J. et al., Stephen's detection and evaluation of adverse drug reactions, 2012).

Drug	Year	Adverse reaction	Outcome
Thalidomide	1961	Congenital malformation	Withdrawn
Chloramphenicol	1966	Blood dyscrasis	Restricted indications
Clioquinol	1975	Subacute myelo-optic neuropathy	Withdrawn
Practolol	1977	Oculomucocutaneous syndrome	Restricted use
Benoxaprofen	1982	Liver damage	Withdrawn
Etomidate	1983	Adrenal supression	Restricted use
Zimeldine	1983	Hypersensitivity	Withdrawn
Indoprofen	1984	Gastrointestinal bleeding/perforation	Withdrawn
Phenylbutazone	1984	Blood dyscrasis	Restricted use
Asprin	1986	Reye's syndrome(children)	Restricted use
Suprofen	1987	Renal impairment	Withdrawn
Spironolactone	1988	Animal carcinoma	Restricted use
Noscapine	1991	Gene toxicity	Withdrawn
Temafloxacin	1992	Allergic reactions and hemolytic anemia	Withdrawn
TGN1412	2005	Cytokine release syndrome	Review of first-in-human studies
Ximelagatran	2006	DILI	Withdrawn
Lumiracoxib	2007	DILI	Withdrawn
Benfluorex	2009	Pulmonary hypertension	Withdrawn
Roziglitazone	2010	Cardiovascular disease	Withdrawn
Lapatinib	2011	DILI	Restricted use
Telaprevir	2012	Cutaneous reactions	Boxed warning

1.1.1 Definition of adverse drug reactions

The World Health Organization (WHO) has defined an ADR as a response to a drug that is noxious, unintended or undesired, occurring at doses normally used for the prophylaxis, diagnosis or treatment of disease (1969). The WHO

definition of ADRs excludes reactions to pharmaceutical excipients and herbal medications. Edwards and Anderson in 2000 criticised the WHO definition. They stated that ADRs can occur at doses other than those described by the WHO definition. They also argued that the word 'noxious' excludes ADRs that may be inconvenient but not harmful. They therefore defined ADR as "an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product which predicts hazard for future administration and warrants prevention, specific treatment, alteration of the dosage regimen or withdrawal of the product" (Edwards and Aronson 2000).

1.1.2 Classification of adverse drug reactions

ADRs can be broadly classified into dose-dependent (type A) and idiosyncratic (type B) reactions (1969; Ahmed et al. 1988; Edwards and Aronson 2000; Riedl and Casillas 2003).

Type A reactions are consistent with the extended pharmacology of the 'culprit' drug and occur at a higher frequency than type B reactions (Einarson 1993). These reactions include toxic effects of drugs such as digoxin and serotonin. Drug overdose, impaired metabolism and compromised excretion are predisposing factors to type A reactions.

Type B reactions are less common, dose-independent, unpredictable and often dependent on individual susceptibility factors. Approximately 10%-15% of all adverse drug reactions are type B reactions (Jick 1984; Kocak et al. 2006). Many type B reactions involve activation of the host adaptive immune system and are therefore often referred to as allergic or hypersensitivity reactions. They

involve different components of the adaptive immune response including IgE antibodies, drug-specific T-cells and immune complexes (Anderson and Adkinson 1987; deShazo and Kemp 1997; Demoly and Bousquet 2001). The major differences between the two types of ADRs are summarised in table 1.2 below.

Table 1.2- Characteristics of type A and type B adverse drug reactions

Type A	Type B
Predictable	Unpredictable
Usually dose dependent	Rarely dose dependent (high mass dose is often implicated)
Low morbidity	High morbidity
Low mortality	High mortality
Responds to dose reduction	Responds to drug withdrawal
Example: e.g. dry mouth associated with tricyclic antidepressants	Example: anaphylaxis with penicillin, skin rashes with antibiotics, agranulocytosis with carbimazole

More recently, strong HLA associations have been linked with certain drug hypersensitivity reactions, thus making them more predictable (Pavlos et al. 2012). Abacavir (Mallal et al. 2002), allopurinol (Hung et al. 2005), carbamazepine (Chung et al. 2004; McCormack et al. 2011) and flucloxacillin (Daly et al. 2009) are among the growing list of drugs with strong HLA-associations (table 1.3).

Table 1.3- Examples of HLA-associated drug hypersensitivity reactions

HLA	Drug	Adverse reaction	HLA association (s)	References
HLA-I association	Abacavir	AHS	B*57:01	Mallal et al., 2002
	Allopurinol	SJS/TEN and HSS	B*58:01	Hung et al., 2005
	Carbamazepine	SJS/TEN	B*15:02 in Asians	Chung et al., 2004
			A*31:01 in Europeans	McCormack et al., 2011
			A*31:01 in Japanese	Ozeki et al., 2011
	Flucloxacillin	DILI	B*57:01	Daly et al., 2009
HLA-II association	Aspirin	Asthma	DPB1*03:01	Dekker et al., 1997
	Hydralazine	Systemic lupus	DR4	Batchelor et al., 1980
	NSAIDs	Anaphylactoid and cutaneous reactions	DR11	Quiralte et al.,1999
	Ximelagatran	DILI	DRB1*07-DQA1*02	Kindmark et al.,2008
Mixed associations	Aminopenicillins	HSS	A2, DRw52	Romano et al., 1998
	Co-amoxiclav	DILI	A*02:01-B*07:02-DRB1*15:01-DQB1*06:02	Lucena et al., 2011
	d-Penicillamine	Proteinuria	B8, DR3	Wooley et al., 1980
	Nevirapine	Cutaneous reactions	Cw4 B*35:05	Likanonsakul et al., 2009 Chantarangsu et al.,2009

ASH-Abacavir hypersensitivity syndrome; SJS-Steven Johnson syndrome,; TEN-Toxic epidermal necrolysis; HSS-Hypersensitivity syndrome; DILI-Drug induced liver injury.

1.1.3 Epidemiology of adverse drug reactions

Type A and B reactions present with complications in drug therapy that may require dose reduction and or withdrawal of the offending medication (Jick 1984). In the United States, it is estimated that 100,000 hospital admissions annually relate directly to drug exposure (Lazarou et al. 1998). In Sweden, ADRs are the 7th most common cause of death (Lavergne et al. 2008). An NHS survey in 2004 revealed that 6.5% of 19,000 hospitalised patients, in two UK hospitals, was as a result of ADRs (Hughes et al. 2004). The significant variability that exists between different ADR epidemiology data may be due to differences in ethnicity, advances in health care, medical practices and study design (Raschetti et al. 1999; Fattinger et al. 2000; Senst et al. 2001; Dormann et al. 2004; Davies et al. 2009)

Risk factors of adverse drug reactions (other than HLA) include: gender; females showing a higher predisposition (Schopf et al. 1991; Barranco and Lopez-Serrano

1998; Beukelman and Mirenda 2005; Sharma et al. 2008) and viral infections; most especially HIV (Bayard et al. 1992; Khambaty and Hsu 2010), and herpes simplex virus (Descamps et al. 2001; Shiohara et al. 2006). The pathophysiological state of the disease and polypharmacy also serve as risk factors to certain ADRs (Lang et al. 1991; Petri and Allbritton 1992; Harb and Jacobson 1993; Atkin and Shenfield 1995; Pirmohamed and Park 2001). Furthermore, the patient and the dose of drug administered are important susceptibility factors (Leach and Roy 1986; van der Ven et al. 1991).

Riedl and Casillas (2003) argued that the chemical properties and molecular weight of a drug constitute the most important risk factor of ADRs (Riedl and Casillas 2003). Drugs with large molecular weight and complex structures like human proteins are more prone to immunological reactions. On the other hand, drugs with low molecular weights (<1000 Daltons) may only become immunogenic by forming antigenic adducts with macromolecules like proteins (Pirmohamed et al. 2002; Holt and Ju 2006; Uetrecht 2007), discussed in greater detail later.

Genetic polymorphisms in drug metabolism enzymes result from mutations in the genes that encode certain enzymes. These mutations may lead to increased, decreased or a total lack of enzyme activity resulting in differential toxicity observed in certain individuals (Meyer and Zanger 1997; Nebert 1997; Tanaka 1999). Polymorphisms in the NAT1 and NAT2 genes that encode for N-acetyltransferase has clinical implications in the metabolism of drugs like caffeine, isoniazid, nitrazepam and sulfonamides (Summerscales and Josephy 2004). Different degrees of toxicity have reported in slow acetylators of dapsone

and procainamide while fast acetylators present with unreliable clinical outcomes with isoniazid (Shenfield 2004). Furthermore, CYP2C9, CYP2C19 and CYP2D6 are highly polymorphic Cytochrome P450 enzymes, involved in either therapeutic failures or adverse drug reactions (Meyer and Zanger 1997; Bozina et al. 2009; Johansson and Ingelman-Sundberg 2011). It is important to state that genetic polymorphism of drug metabolism enzymes and immune reactions have been studied extensively and there is no correlation. Figure 1.1 summarises the major risk factors that predispose a patient to ADRs.

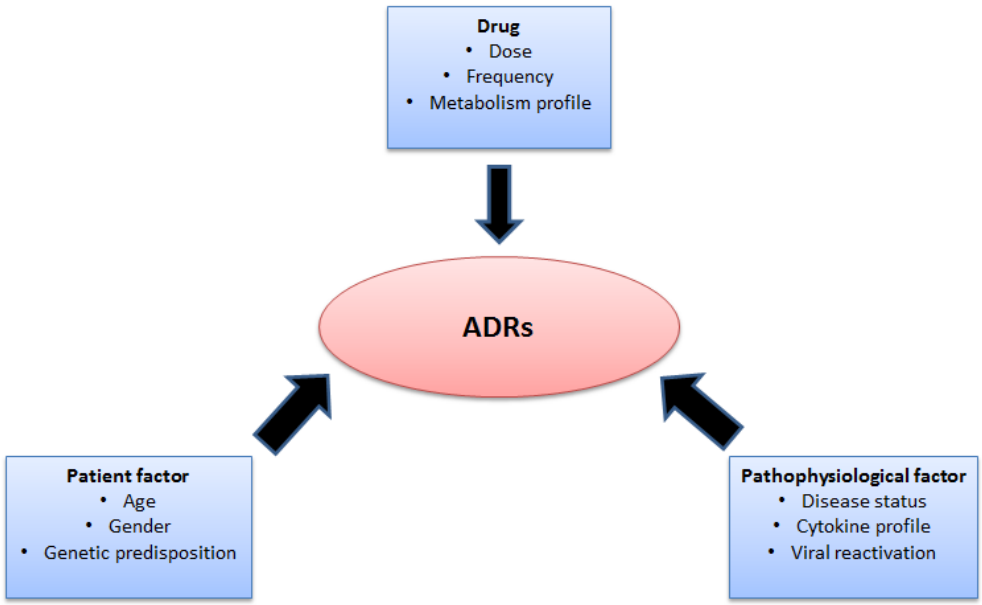


Figure 1.1- Schematic representation of the risk factors involved in ADRs

1.1.4 Clinical and economic impact of adverse drug reactions

ADRs are common causes of hospital admissions worldwide significantly, straining the healthcare system and resulting in many deaths (Bergman and Wiholm 1981; Hallas et al. 1990; Leape et al. 1991; Suh et al. 2000; Patel et al.

2007; Brvar et al. 2009). In the UK, extended hospital admission resulting from ADRs is estimated to be eight days and accounts for approximately 4% of the total capacity of hospital beds, costing the NHS about £466m (Pirmohamed et al. 2004). Common causes of "inadvertent" ADRs include missed doses, illegible orders, wrong techniques, duplicate therapy, drug-drug interactions, preparation error, equipment failure and inadequate monitoring (Leape et al. 1991; Evans et al. 1994; Lesar et al. 1997).

1.1.5 Definition of drug hypersensitivity reactions

Drug hypersensitivity reactions are defined as ADRs with an immunological aetiology to an otherwise safe and effective therapeutic agent, administered at recommended doses. They are widespread and pose a serious public health challenge. Recent advances in the field of drug hypersensitivity research have enhanced our understanding of the complex pathophysiology of drug hypersensitivity reactions. They are immune-mediated reactions presenting with mild to severe symptoms including skin rash, anaphylaxis and serum sickness. SJS and TEN are among the very severe skin manifestations of drug hypersensitivity reactions (Pohl et al. 1988; Gomes and Demoly 2005).

1.1.6 Time course of drug hypersensitivity reactions

The time course for the appearance of clinical symptoms of hypersensitivity reactions is important in their diagnosis, but also in the treatment of these reactions. Many drug hypersensitivity reactions present as delayed-type reactions and require between one day to a few weeks for clinical manifestations to appear (Hausmann et al. 2010). Activation of T-cells has been reported as a key feature of drug hypersensitivity reactions (Naisbitt et al. 2003;

Wu et al. 2006; Castrejon et al. 2010). Molecular changes that accompany antigen-specific T-cell activation include T-cell commitment (0-30 minutes post exposure to antigen) and T-cell proliferation (1-2 days post antigen activation) (Stepp et al. 2000; Posadas et al. 2002). Terminal functional differentiation involving the production of cytotoxic molecules like perforin and granzyme-B occurs 5-7 days post antigen stimulation (Ullman et al. 1990; Ortiz et al. 1997). Drug-induced liver injury (DILI) is a low incidence, but serious complication of a number of drugs including lapatinib, lumiracoxib and ximelagatran (see table 1.1). The mean onset of action of DILI with flucloxacillin and co-amoxiclav is 110 days (Andrade et al. 2006). Although HLA association suggests an immune-mediated mechanism, the reason for this time course is unknown.

1.1.7 Classifications of hypersensitivity reactions

Gell and Coombs (1963) classified hypersensitivity reactions and other immune-mediated reactions into four major categories. The classification is based on the time to clinical symptoms, and mechanistic features of the reactions. The four groups include: type I, type II, type III and type IV reactions (table 1.4).

Type I reactions are mostly IgE-mediated reactions and result from cross-linkage of receptors on mast cells and basophils leading to an immediate release of histamine and leukotrienes. Clinical manifestations include bronchoconstriction, urticaria, eczema, conjunctivitis and anaphylaxis.

Type II reactions are also known as cytotoxic reactions and are mediated by IgG or IgM antibodies, which bind to cells followed by binding of complement proteins and cell rupture. This mechanism is responsible for the clinical

manifestations of haemolytic anaemia, granulocytopenia and thrombocytopenia.

Type III reactions are either IgG or IgM mediated reactions occurring in the presence of an excess concentration of an antigen in the circulation. Examples of type III reactions include serum sickness, systemic lupus erythematosus, rheumatoid arthritis and vasculitis.

Type IV reactions are T-cell mediated and mostly delayed-type hypersensitivity reactions. This mechanism is involved in the pathogenesis of a number of autoimmune and infectious diseases such as tuberculosis, leprosy, blastomycosis, histoplasmosis, toxoplasmosis, and leishmaniasis. Stevens-Johnson syndrome and toxic epidermal necrolysis are examples of type IV reactions. Table 1.4 compares the four types of hypersensitivity reactions classified according to Gell and Coombs.

Table 1.4- Comparison of the different mechanisms of hypersensitivity reactions

Characteristics	Type I (anaphylactic)	Type II (cytotoxic)	Type III (immune-complex)	Type IV (delayed type)
Antibody	IgE	IgG, IgM	IgG, IgM	None
Antigen	Exogenous	Cell surface	Soluble	Tissues & organs
Response time	15-30 minutes	Minutes-hours	3-8 hours	48-72 hours
Histology	Basophils & eosinophils	Antibody & complement	Complement & neutrophils	Monocytes and lymphocytes
Appearance	Weal & flare	Lysis & necrosis	Erythema, edema & necrosis	Erythema& induration
Mediators	Antibody	Antibody	Antibody	T-cells
Examples	allergic asthma, hay fever	erythroblastosis fetalis, Goodpasture's nephritis	SLE, farmer's lung disease	tuberculin reaction, poison ivy, granuloma
Drugs implicated	Penicillin, cephalosporin	Penicillin	Penicillin	Sulphamethoxazole, flucloxacillin

The majority of drug-induced hypersensitivity reactions are delayed type IV reactions and can be further sub-divided into four classes (IV a, b ,c and d); with monocyte, eosinophil, cytotoxic T-lymphocyte and neutrophil involvement in the respective classes (Depta et al. 2004). Table 1.5 illustrates the main features of the various sub-types of class IV hypersensitivity reaction.

Table 1.5- Characteristics of type IV hypersensitivity reaction

Sub-type	T-cell effector molecule	Pathophysiology	Examples
IV a	Interferon gamma	Monocytic inflammation	Eczema
IV b	IL-4, IL-5	Eosinophilic inflammation	Maculopapular exanthema (MPE)
IV c	Perforin, Granzyme B	Keratinocytes death mediated by CD4+ and CD8+ T-cells	Bollus exanthema (BE) and MPE
IV d	CXCL-8, IL-8	Neutrophilic inflammation	Pustular exanthema (PE), MPE and BE.

1.1.8 Diagnosis of drug hypersensitivity reactions

Skin tests are the only tests used in routine clinical diagnosis of ADRs. The guidelines for carrying out skin tests in the diagnosis of drug-induced cutaneous reactions have been reported by a number of studies (Barbaud et al. 2001; Brockow et al. 2002; Aberer et al. 2003). Traditional skin tests such as the prick, patch and intracutaneous tests are disadvantaged by their low sensitivity (sometimes false-negative) and their invasive nature (Barbaud et al. 1998; Barbaud et al. 2001; Strauss et al. 2001; Torres et al. 2003; Romano et al. 2004). Challenge tests are sometimes useful to confirm inconclusive results from prick and patch tests. This may result in the reactivation of severe symptoms and fatality, hence must be carried out under close medical observation. In severe reactions, challenge tests are discouraged (Aberer et al. 2003; Han et al. 2012).

The diagnosis of drug-induced delayed-type T-cell mediated hypersensitivity reactions is important in order to characterise clinical symptoms of these reactions and establish the drug (s) responsible for such reactions. Drug-specific T-lymphocytes have been isolated from blister fluid, skin tissues and peripheral blood of hypersensitive patients and may remain detectable years after the drug was administered (Hari et al. 2001; Naisbitt et al. 2005; Beeler et al. 2006).

In vitro techniques provide safer alternatives to the often invasive skin tests in the diagnosis of drug-hypersensitivity reactions and offer a better understanding of the molecular mechanism of these reactions. The sensitivity of most *in-vitro* tests is less than 100%; hence accurate patient history is an important complementary data for accurate diagnosis. These tests include

- Lymphocyte transformation test (LTT)
- Enzyme-linked immunospot (ELISPOT) assay
- Others (Flow cytometry, intracellular cytokine staining, multiplex assays and T-cell cloning)

Importantly, these tests are currently not used in routine clinical care due to a number of reasons: (1) these assays rely on the availability of freshly isolated peripheral blood mononuclear cells and/or a cryopreservation facility, if assays are to be conducted on a later date. (2) A researcher must be trained in cell isolation, sterile technique and cell culture methods. (3) Incorporation of the drug into the assay in an appropriate form is technically demanding.

1.1.8.1 Lymphocyte transformation test (LTT)

LTT is also referred to as lymphocyte stimulation test or lymphocyte proliferation test and is the most utilised *in vitro* assay for the diagnosis of penicillin allergy (Nyfeler and Pichler 1997; Luque et al. 2001; Pichler and Tilch 2004). It measures the proliferation of memory T-lymphocytes to a particular drug to which a patient has been exposed and provides an insight into the pathomechanism of these reactions (Nyfeler and Pichler 1997).

The LTT can be utilised for both delayed-type and immediate hypersensitivity reactions (Luque et al. 2001). It has a high specificity with various drugs including the β-lactams (amoxicillin, flucloxacillin, and piperacillin), sulfamethoxazole (SMX), lidocaine, celecoxib, lamotrigine, ciprofloxacin, carbamazepine, mepivacaine and *p*-phenylenediamine. The sensitivity of LTT is dependent on the chemical properties of the drug involved (Gerber and Pichler 2004; Pichler and Tilch 2004; Romano et al. 2004).

The assay is performed using peripheral blood mononuclear cells (PBMCs) isolated from sensitised patients over a density gradient. Proliferative response can be evaluated using [^{3}H]-thymidine incorporation during DNA synthesis. Lymphocyte proliferation is expressed as stimulation index (SI), which is the ratio of antigenic proliferation against proliferation in the presence of cell culture medium. A stimulation index of ≥ 2 is considered as a positive response. LTT data for immunosuppressive drugs e.g corticosteroids, known to interfere with lymphocyte proliferation should be interpreted relative to both the positive and the negative controls (Pichler and Tilch 2004). Finally, regardless of the advantages of the LTT as a non-invasive assay, the use of radioactive isotopes and long duration of the assay (6 days) are obvious disadvantages in terms of clinical applications.

1.1.8.2 Enzyme-linked immunospot (ELISPOT) assay

ELISPOT assay is a quick, highly sensitive and precise technique applied in the detection and characterization of low frequency drug-specific cytokine-secreting T-lymphocytes (Czerkinsky et al. 1988; Jenkins et al. 2013; Monshi et al. 2013). This type of assays provides an insight into the biological function of the T-lymphocytes that mediate drug hypersensitivity reactions. Activated T-lymphocytes can secrete an array of pro-inflammatory (Th1) cytokines (IFN-gamma, TNF-α, IL-2, IL-6 IL-12 and IL-13) and anti-inflammatory (Th2) cytokines (IL-4, IL-5, IL-10 and TGF-β) as well as other effector molecules like granzyme, perforin and Fas ligand (Romagnani 1992; Aberer et al. 2003; Lehmann and Zhang 2012).

IFN-ϒ is an important cytokine implicated in a number of delayed-type drug hypersensitivity reactions; therefore, the number of circulating drug-specific

IFN-ϒ secreting T-lymphocytes has been quantified as a diagnostic measure of drug-induced hypersensitivity reactions (Pichler 2003; Teraki and Shiohara 2003; Yoshimura et al. 2004; Naisbitt et al. 2005; Rozieres et al. 2009). Besides serving as a marker for cytotoxicity, raised levels of IFN-ϒ is known to upregulate MHC class II molecules on the surface of keratinocytes resulting in an enhanced antigen presentation to $CD4^+$ T-lymphocytes (Yawalkar et al. 2000; Beeler and Pichler 2006). A sensitivity of 90% has been reported using IFN-ϒ specific ELISPOT in the detection of penicillin-specific T-lymphocytes in patients with maculopapular exanthema (Rozieres et al. 2009).

1.1.8.3 Flow cytometry

Flow cytometry is a vital technique used in the identification of multiple phenotypic cell surface parameters. Cell surface markers of T-cell activation on peripheral blood can be used to detect antigen-specific T-cell populations and subsequently in the diagnosis of an antigen-specific T-cell response and hypersensitivity (Hari et al. 2001). For example, the upregulation of CD69 on $CD4^+$ T-cells in response to antigenic stimulation has previously been used in the detection of phenytoin-specific T-lymphocytes (Lochmatter et al. 2008). Other cell surface markers of T-cell activation include CD25, CD40L, CD71 and HLA-DR (Beeler and Pichler 2006). The upregulation of these cell surface markers is drug-specific and does not occur in non-sensitized individuals.

Flow cytometry can also be used to evaluate proliferative response following stimulation of memory T-lymphocytes. Antigen-specific T-lymphocytes are first stained with the fluorescent dye, carbooxyfluorescin diacetate succinimidyl ester (CFSE), which interacts with the amino group of intracellular proteins. The intensity of CFSE is reduced by half following each cell division resulting from

drug stimulation. Hence, the dividing population of drug-specific T-lymphocytes can be evaluated.

1.1.8.4 Intracellular cytokine staining (ICS)

ICS is a flow cytometry-based assay that evaluates the intracellular cytokine secretion by activated cells. The nature of an immune response following an antigen stimulation is thought to be defined by a fine balance between Th1, IFN-ϒ secreting cells and Th2, IL-4 secreting cells (Del Prete et al. 1993; Kroemer et al. 1993). Both subsets of T-cell have no distinguishing cell surface markers hence, ICS for both cytokines using fluorescent antibodies followed by flow cytometry can serve as a diagnostic tool to evaluate their involvement in drug-induced reactions. ICS can detect multiple cytokines secreted by a sub-set of lymphocytes following antigen stimulation.

1.1.8.5 Multiplex (Luminex) assays

Like the ELISPOT assay, the multiplex assay quantifies cytokines secreted following T-lymphocyte stimulation by drugs or metabolites (Beeler and Pichler 2006; Chen et al. 2009; Elsheikh et al. 2011). This assay utilises beads with known spectral characteristics that are bound to particular capture antibodies in order to detect and quantify cytokines secreted by defined cell populations.

1.1.8.6 T-cell cloning

In a limited number of individuals, it is difficult to detect drug-specific T-lymphocytes using the previously described assays. Hence, T-cell cloning provides a valuable alternative. T-lymphocyte cloning can be performed according to the well-established method of 'limiting dilution' (Staszewski 1984). T-cell clones can then be characterised in terms of phenotypic marker or proliferation and cytokine secretion following drug-stimulation. T-cell cloning is

an important technique used to evaluate the pathomechanism of drug hypersensitivity reactions.

1.1.9 Cutaneous drug reactions

Drug-induced hypersensitivity reactions target multiple organs within the human body including lungs, heart, liver, kidneys and skin. However, the skin is the most commonly affected organ and accounts for 2-3% of adverse drug reactions in hospitalised patients (Bigby et al. 1986; Hunziker et al. 1997; Crowson et al. 2003). The large surface area, dense network of dendritic cells (DCs) and vast network of blood vessels make the skin more susceptible to pathogenic T-cell reactions compared with other organs (Keller et al. 2005; Clark et al. 2006).

Cutaneous reactions to medications vary in appearance and severity. SJS and TEN are the most severe forms, with higher mortality rates compared to maculopapular exanthema (MPE), acute generalised exanthematous pustulosis (AGEP), and drug rash/reaction with eosinophilia and systemic symptoms (DRESS). Other cutaneous manifestations of drug hypersensitivity reactions include urticaria, fixed drug eruptions, photosensitive reactions and erythema exudativum multiforme (Bigby et al. 1986; Hunziker et al. 1997; Crowson et al. 2003).

The clinical manifestations of the different cutaneous adverse drug reactions differ in terms of cytokines secreted by activated T-lymphocytes. While $CD4^+$ T-lymphocytes cells play an important role in drug induced MPE and AGEP, $CD8^+$ lymphocytes are involved in SJS and TEN (Nassif et al. 2004). A large number of

drugs belonging to different pharmacological classes cause cutaneous reactions (table 1.6).

Table 1.6- Drugs commonly associated with cutaneous hypersensitivity reactions

SJS	AGEP	DRESS
Nevirapine Allopurinol Phenytoin Carbamazepine Lamotrigine Cotrimoxazole Barbiturates NSAIDs (Oxicams) Setraline Pantoprazole Tramadol	Aminopenicillins Cephalosporins Pristinamycin Celecoxib Quinolone Diltiazem Terbinafine Macrolides	Carbamazepin Phenytoin Lamotrigine Dapsone Minocyclin Sulfasalazine Cotrimoxazole Abacavir

The following sections summarise the clinical manifestations and causes of the different forms of drug-induced skin reactions.

1.1.9.1 Maculopapular exanthema (MPE).

MPE is the most common cutaneous manifestation of the beta-lactam, SMX, quinolone, NSAID, anticonvulsants and allopurinol hypersensitivity. However, there are several other causes of MPE including HIV, EBV, and CMV, which complicate diagnosis (Fernandez et al. 2009). MPE accounts for between 31-95% of all drug-induced skin reactions (Apaydin et al. 2000; Bigby 2001). The onset of MPE is between 8-11 days following drug administration and continues

for up to 2 days after discontinuation of the offending drug (Valeyrie-Allanore et al. 2007).

Clinical manifestations of MPE include faint, pink or red macules that further develop into maculopapular rash, moderate to severe itching and fever. Drug-specific cytotoxic $CD4^+$ T-lymphocytes are the predominant effector cells that mediate MPE (Hertl and Merk 1995; Blanca et al. 2000; Yawalkar et al. 2000; Pichler et al. 2002; Bronnimann and Yawalkar 2005; Yawalkar 2005). Secretory molecules including INF-ϒ, perforin, granzyme-B and IL-5 play an important role in the pathophysiology of MPE (Schnyder et al. 1998; Posadas et al. 2000; Yawalkar et al. 2000; Posadas et al. 2002). Chemokines like CCL11/eotaxin, CCL5/RANTES, and CCL27/CTACKS also mediate MPE (Yawalkar et al. 2000; Tapia et al. 2004). Generally, supportive treatments for mild MPE include withdrawal of the offending drug and application of emollients to affected areas. For more severe MPE reaction, short courses of systemic anti-histamines and topical corticosteroids are administered.

1.1.9.2 Acute generalised exanthematous pustulosis (AGEP)

AGEP is an uncommon cutaneous reaction first described in 1980 by Beylot and his colleagues (Beylot et al. 1980). It is also referred to as pustular drug eruption or toxic pustuloderma and is mediated by IL-8 secreting T-lymphocytes. Antibiotics including aminopenicillins, macrolides, clindamycin and sulfonamides are the major drugs implicated in AGEP (Schmid et al. 2002; Sidoroff et al. 2007). The incidence of AGEP is between 1-5 per million per year (Sidoroff et al. 2001; Leclair et al. 2009). The onset of AGEP is about five days following the administration of the causative drug, with clinical symptoms persisting for 1-2 weeks after discontinuation of drug therapy (Zlotnik and

Yoshie 2000). AGEP is characterised by numerous small primary, non-follicular sterile pustules, leukocytosis, eosinophilia and large areas of edematous erythema (Roujeau et al. 1991; Roujeau 2000; Britschgi et al. 2001). Skin detachment similar to that seen in SJS and TEN has also been reported (Roujeau 2000; Peermohamed and Haber 2011). In the absence of appropriate clinical intervention, the mortality from AGEP is about 5 percent (Roujeau 2005).

1.1.9.3 Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS)

DRESS is also referred to as drug hypersensitivity syndrome and is characterized by high fever, morbilliform skin rash, malaise, lymphadenopathy and damage to multiple internal organs (kidneys, liver, lungs, and/or heart). The liver is the organ most commonly involved, often resulting in fulminant hepatitis (Amante et al. 2009). The pathophysiology of DRESS involves the recruitment of eosinophils, mediated through IL-5 secreted by activated T-lymphocytes (Choquet-Kastylevsky et al. 1998). The reactivation of human herpes virus 6 and 7 (HHV-6 and HHV-7) plays a critical role in DRESS (Descamps et al. 1997; Kano et al. 2004). Certain clinical manifestations of DRESS are linked to a systemic immune response against reactivation of HHV-6, HHV-7, Epstein-Barr virus and cytomegalovirus, prompted by the causative drug (Picard et al. 2010). Picard and his colleagues reported viral reactivation in 76% of patient with DRESS following administration of carbamazepine, allopurinol, or SMX. Interestingly, they reported that approximately 50% of the $CD8^+$ T-lymphocytes involved responded to a number of EBV epitopes. Although systemic manifestations of DRESS are $CD8^+$ T-lymphocyte-mediated, often targeting herpes viruses; the role of the drug and viral specific T-cells in the

disease pathogenesis is yet to be defined. These reactions are delayed and T-cell mediated with onset between 2-8 weeks after administration of the offending drug(s) (Peyriere et al. 2006). DRESS has an estimated mortality of 8%.

Allopurinol, carbamazepine, phenobarbital, phenytoin, lamotrigine and the sulfonamides antibiotics are common drugs associated with DRESS. Aromatic anticonvulsants are the most common class of drugs implicated in DRESS (Gaedigk et al. 1994; Edwards et al. 1999; Bohan et al. 2007). Aromatic hydroxylation results in the formation of arene oxide (toxic reactive intermediates) normally transformed to nontoxic metabolites by microsomal epoxide hydroxylase or glutathione transferase. Other arene oxide detoxification pathways involve conversion to nontoxic phenol derivatives (Shear and Spielberg 1988; Gogtay et al. 2005). Failure to detoxify arene oxide results in covalent modification of macromolecules that form the antigenic determinants responsible for T-cell mediated hypersensitivity reactions (Gennis et al. 1991; Gaedigk et al. 1994; Krauss 2006).

The diagnosis of DRESS relies on the clinical presentation of a combination of symptoms, namely high fever, skin rash and organ involvement accompanied by eosinophilia. Accurate diagnosis of the offending drug also relies on a carefully documented history of drug intake, and may be complicated by the varied time to onset (2-8 weeks) after drug exposure. Immediate withdrawal of drug(s) and supportive symptomatic care are the first line of treatment for DRESS. Antihistamines, systemic steroids and immunoglobulin therapy are used to treat more severe cases.

1.1.9.4 Stevens Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN)

The incidence of SJS is 6 cases in a million people per year and 2 cases per million in a year for TEN (Roujeau and Stern 1994; Rzany et al. 1996). Hence, both conditions are extremely rare. Nevertheless, SJS and TEN are life-threatening skin conditions accompanied by keratinocyte cell death and exfoliation of the epidermis (Roujeau and Stern 1994; Yang et al. 2007; Mockenhaupt 2011). TEN has been described as a more severe form of SJS.

The degree of separation of the epidermis from the dermis is the major clinical diagnostic feature for SJS and TEN (Roujeau and Stern 1994; Ward et al. 2010). Skin detachment of 10% or less is consistent with SJS, 10-30% in SJS-TEN and greater than 30% in TEN (Roujeau 1994; Sharma and Sethuraman 1996; Mockenhaupt 2009). Classes of drug that cause SJS/TEN include antibiotics (co-trimoxazole, penicillins, cephalosporins), antifungal (imidazole), antiviral (nevirapine) and non-steroidal anti-inflammatory drugs (naproxen, ibuprofen), anti-convulsants (carbamazepine, phenytoin, phenobarbital, valporic acid and lamotrigine) and allopurinol (Roujeau 1994; Rzany et al. 1996; Halevy et al. 2008; Sharma et al. 2008; Khambaty and Hsu 2010; Ward et al. 2010). These conditions are accompanied by general malaise, fever and multi-organ involvement (Greenberger 2006; Feldmeyer et al. 2010; Harr and French 2010; Harr and French 2010).

The pathogenesis of SJS/TEN is still poorly understood despite the high mortality rate of between 10-50% (Gomes and Demoly 2005). Cytotoxic T-lymphocytes and natural killer T-lymphocytes have been implicated in SJS and TEN, causing marked keratinocyte apoptosis (Paul et al. 1996; Ko et al. 2011;

Wang et al. 2013). Recent research into the pathomechanism suggests that cytotoxic T-lymphocytes are activated by offending drugs presented on keratinocytes in an MHC class I restricted manner (Gao and Jakobsen 2000; Wei et al. 2012).

Activated cytotoxic T-lymphocytes secrete cytotoxic proteins, cytokines and chemokines that mediate the clinical outcomes of drug induced SJS/TEN (Yang et al. 2007; Ko et al. 2011). The pathogenesis of SJS/TEN involves two major pathways of cell death, the Fas ligand pathway and perforin/granzyme-B pathway (Viard et al. 1998; Nassif et al. 2002; Posadas et al. 2002; Abe et al. 2003; French 2006; Khalili and Bahna 2006; Czarnobilska et al. 2007; Torres et al. 2009). Furthermore, Chung et al (2008) have demonstrated the presence of T-lymphocytes and NK cells in the blister fluids from patients with SJS/TEN. Blister fluids contained 2-4 fold higher expression of granulysin when compared with perforin, Fas ligand and granzyme-B (Chung et al. 2008).

The expression of several HLA molecules has been associated with the development of SJS/TEN (Chen et al. 2011; Sanchez-Giron et al. 2011; Somkrua et al. 2011). Importantly, these HLA associations (discussed in greater detail later in the thesis) are often drug-specific and often restricted to certain ethnic groups.

Although no standard treatment exists for SJS/TEN, the use of intravenous immunoglobulin therapy (Viard et al. 1998; Teo et al. 2009) and corticosteroids (Fromowitz et al. 2007) have been reported to show clinical benefit. Paradoxically, a detailed review of 156 patients who received high dose intravenous immunoglobulin suggested no obvious efficacy (Faye and Roujeau

2005). Furthermore, a novel peptide therapy targeting CD8 surface receptors with DNA aptamer conjugated with granulysin siRNA was effective in decreasing immune responses by cytotoxic lymphocytes and the disease pathogenesis (Wang et al. 2013).

1.2 Immune system

The ability to recognise and respond to antigens is the fundamental basis of the immune system. An immune response is defined as a reaction to any component of a microbe or macromolecule. The first documented reference of immunology dates back to 430 BC during the plague of Athens (Retief and Cilliers 1998). Louis Pasteur and Robert Koch were among the early contributors to the field of immunology (Plotkin 2005; Stanisic et al. 2010). Louis Pasteur's discovery that the immune system could be modulated by attenuated microbes or microbial products remains the fundamental principle of vaccination. On the other hand, Robert Koch proposed that acquisition of immunity to malaria required constant exposure to the plasmodium parasite over a number of years.

The most important physiological function of the immune system is protection from microbial and viral infections and foreign molecules. Protection against foreign organisms is mediated in the early stages by the innate immune system and further complemented by the adaptive immune system. Gene recombination ensures that large diversities of T-cell receptors are expressed to encounter unlimited antigenic epitopes (Market and Papavasiliou 2003).

The bone marrow and thymus are the origin of all immune cells found in the blood, spleen and lymphatic tissues, and are responsible for immune

surveillance. The different sub-sets of T-lymphocytes and their involvement in cell-mediated immunity will be discussed in detail.

While the innate immune system is non-specific, responding to a broad class of foreign stimuli (proteins, carbohydrates, nucleic acids and microbial structures) secondary to tissue damage and/or microbial infections, the adaptive immune system is specific and dependent on secreted antibodies, phagocytes and cytotoxic T-lymphocytes for immune protection. The following sections summarise the role of the innate and adaptive immune system in the maintenance of tissue homeostasis.

1.2.1 Innate Immunity

The innate immune system consists of physiochemical barriers (epithelial layer), chemical mediators (defensin), lysosomes and phospholipids. Inflammatory mediators, cytokines, coagulation cascades and the complement system make up the humoral barrier while the cellular components include phagocytes (neutrophils, macrophages dendritic cells), eosinophils and natural killer cells. The innate immune system blocks the access of micro-organisms and destroys or limits their proliferation within the tissues they invade.

Recognition of pathogen-associated molecular patterns (PAMPs) represents one of the major important molecular mechanisms of the innate immune response (Janeway 1989; Janeway 2000). These receptors are fixed in the genome, non-clonal and are called pathogen recognition receptors (PRRs). PRRs can be either soluble or cell-associated and expressed on innate immune cells like dendritic cells, macrophages and neutrophils (Kumar et al. 2009; Kawai and Akira 2010; Takeuchi and Akira 2010). The interaction between soluble PRRs and PAMPs

may result in the direct attack of a microorganism by soluble PRR molecules, enhanced phagocytosis of PRR-bond PAMPs and lysis of microorganisms. On the other hand, interaction of PAMPs with cell-associated PRRs results in the phagocytosis of PAMP-associated microorganisms and activation of intracellular signalling pathways that release inflammatory mediators.

Tissue damage and non-physiological death (necrosis) produce certain molecules referred to as danger-associated molecular patterns (DAMPs) capable of activating the immune system (Bianchi 2007; Kono and Rock 2008).

1.2.1.1 Cellular component of the innate immune system

The cells of the innate immune system provide the second level of immunological defence to infections, after the physical barrier provided by the skin. The responses of innate immune cells are immediate and directed towards eliminating infectious agents as well as stimulating the adaptive immune system. These cells include neutrophils, eosinophils, basophils, mast cells, macrophages, monocytes, natural killer cells and dendritic cells. The details of the innate immune cells are summarised below.

Natural killer (NK) cells are $CD56^{+}$ lymphocytes that lack antigen-specific cell surface receptors but produce cytokines that are vital to pathogen and tumour immunity. NK cells mature in the bone marrow, lymph node, spleen, tonsils and thymus and migrate to the blood, spleen, liver and lungs (Yokoyama et al. 2004; Di Santo 2006; Iannello et al. 2008). They make up one-third of all cells originating from the common lymphoid progenitors that generate B- and T-lymphocytes.

NK cell killing is mediated by perforin and granzyme pathways. Activated NK cells secrete IFN-ϒ which enhances the microbicidal activities of macrophages and also stimulates IL-12 production known to polarise Th1 lymphocytes. Although target recognition and cytokine stimulation are the two major mechanisms of NK activity, MHC I specific inhibitory receptors on NK cells play an important role in immune response (Herberman et al. 1975; Kiessling et al. 1975; Yokoyama et al. 2004; Di Santo 2006; Terunuma et al. 2008). Following receptor activation, NK cells produce cytokines like IFN-ϒ, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF) which facilitate the destruction of infected cells (Anegon et al. 1988; Arase et al. 1996; Smith et al. 2002).

Granulocytes or polymorphonuclear leukocytes are a sub-class of leukocytes that contain toxic chemicals capable of digesting microorganisms by phagocytosis. Examples include neutrophils, eosinophils and basophils. Neutrophils make up about 70% of leukocytes found in blood and are involved in phagocytosis. They are granulocytes that contain acidic lysosomes, lactoferrin and myeloperoxidase. Neutrophil migration to infected tissues is mediated by IL-8 (Huber et al. 1991; Smart and Casale 1994; Shen et al. 2006). Upon entry into infected tissues, neutrophils engulf and destroy microorganisms. Eosinophils are granulocytes that secrete IL-2, IFN-ϒ, IL-4, IL-5 and IL-10. They contain toxic cationic granules essential for the destruction of pathogenic parasites. Eosinophil recruitment to sites of inflammation is mediated by IL-5 and eotaxin. Basophils are the least common of the granulocytes and are made up of granules containing pro- inflammatory mediators such as histamine, IL-4

and leukotrienes. They resemble tissue mast cells in terms of histamine- and IgE-mediated responses.

Macrophages are antigen presenting cells (APCs) derived from blood monocytes and are responsible for the phagocytosis of cellular debris and pathogens in both the innate and adaptive immune response. Macrophages make up about 10-15% of the total cell number in some areas of the body and express CD14, CD40, CD11b, CD64 and CD68 (Khazen et al. 2005). They possess an array of PRRs and endosomes that enable them to engulf and kill microorganisms. Endotoxin and IFN-ϒ can activate macrophages to phagocytose and destroy microorganisms. Reactive oxygen species, nitric oxide and lysosomal enzymes are critical components of microbial killing by macrophages. When activated, macrophages present antigens to helper T-lymphocytes resulting in the secretion of pro-inflammatory cytokines. The extrahepatic oxidation of certain drugs by myeloperoxidase (MPO) has been reported to occur in neutrophils and macrophages (Uetrecht 1989; Uetrecht 1992).

Dendritic cells (DCs) are also known as professional APCs. They migrate to the tissues where they reside and continuously survey their environment for infections and tissue damage. The DCs that are resident in skin are referred to as Langerhans' cells. Like macrophages, DCs possess PRRs and Toll-like receptors for immune surveillance.

Antigen presentation by DCs involves internalization of protein antigens, which are processed into smaller fragments and presented on the major histocompatibility complex (MHC) for recognition by T-lymphocytes. Immature DCs express low-level of MHC class II and costimulatory molecules (CD80 and

CD86), they are less mobile and have high phagocytic capacity (Spaggiari et al. 2009). DC maturation is also enhanced by signals from bacterial components and PAMPs such as lipopolysaccharide (LPS), and is associated with the release of DAMPs such as HMGB1, heat shock proteins and uric acid (Shi et al. 2003). Immature DCs are mostly found in epidermal tissues where contact with antigens is most likely.

Following interaction with an antigen, immature DCs migrate to regional lymph nodes where they prime naive T-lymphocytes resulting in a population of memory T cells (Jacob and Baltimore 1999; Banchereau et al. 2003). Direct activation of DCs by haptens and metal ions takes place through the activation of MAP kinase- and NF-κB-dependent pathways (Martin et al. 2006). Unlike mature DCs, antigen presentation by immature or partially mature DCs is known to result in an expansion of regulatory T-lymphocytes and subsequent immune tolerance (Gallucci and Matzinger 2001).

T-lymphocyte activation by DCs is based on two signals, namely; an antigenic signal and an accessory or co-stimulatory signal which is antigen non-specific (Gimmi et al. 1991; Jenkins et al. 1991). The immunostimulatory function of DCs is enhanced by cytokines like IL-4 and GM-CSF. Finally, DCs possess the capacity to locally metabolise certain drugs. They have been reported to express a number of drug metabolism enzymes such as myeloperoxidase (Sanderson et al. 2007). Localised generation of drug reactive metabolites in organs like the skin may be responsible for their high susceptibility to ADRs

1.2.2 Adaptive immune system

The adaptive immune system is also referred to as acquired or specific immune system. The main function of the adaptive immune system is to destroy invading pathogens and their toxic products (Lamond 2002). Complex cellular and molecular interactions exist between the innate and adaptive immune system (Cooper et al. 2004; Raulet 2004); with the innate immune system capable of activating the adaptive immune responses. Immunological memory of the adaptive immune system ensures that a much faster response is obtained upon contact with a previously encountered antigen (Pancer and Cooper 2006; Tokoyoda et al. 2010; Zielinski et al. 2011).

1.2.2.1 Cellular component of the adaptive immune system

The immune system consists of a conglomerate of cells and molecules tasked with antigen recognition and elimination. This section summarises the different cells of the adaptive immune system, their origin, differentiation, activation pathway(s) and functions. Lymphocytes are the major cellular component of the adaptive immune response. They mediate both cellular and humoral immunity (Miller and Osoba 1967; Herzenberg 2000). They originate from hematopoietic stem cells found in the bone marrow. These cells proceed through distinct differentiation pathways to maturation in either the thymus or the bursa cavity (Engel and Murre 2002; Greenbaum and Zhuang 2002; Schebesta et al. 2002). Based on the mechanism of antigen recognition and immune function, lymphocytes can be broadly classified as B-lymphocytes and T-lymphocytes. Activated T-lymphocytes differentiate into cytokine-secreting effector cells while activated B-lymphocytes differentiate into antibody-secreting plasma

cells. The subclasses and immune function of lymphocytes are discussed in more detail in the following sections.

1.2.2.1.1 T-lymphocytes

T-lymphocytes function mainly to generate a specific response against 'non-self' antigens presented on APCs (Zinkerna.Rm and Doherty 1974). They are broadly classified into two functionally distinct groups, helper T-lymphocytes ($CD4^+$) and cytotoxic T-lymphocytes ($CD8^+$). Although some cytotoxic T-lymphocytes are dependent on $CD4^+$ lymphocytes for the development of efficient memory, majority of them respond independently of $CD4^+$ T-cells (Kast et al. 1986; Nash et al. 1987; Ahmed et al. 1988; Husmann and Bevan 1988; Liu and Mullbacher 1989; Bennett et al. 1997).

T helper (Th) lymphocytes are involved in a number of vital processes including B-lymphocyte antibody class switching and activation of cytotoxic T-lymphocytes, phagocytes and macrophages (Behrens et al. 2004). IL-2 and IL-4 regulated by STAT 4 (signal transducer) and STAT 6 (activator of transcription 4), respectively, play vital roles in the differentiation of T helper lymphocytes (Le Gros et al. 1990; Swain et al. 1990; Hsieh et al. 1992; Seder et al. 1992).

All T-lymphocytes express cell surface receptors called T-cell receptors (TCRs) that recognise peptide antigens presented on host proteins encoded by genes in the MHC. The interaction of TCR with MHC-peptide complexes result in clonal expansion and subsequent programmed differentiation of helper T-lymphocytes which gives rise to a highly polarised immune response in the event of an infection (Abbas et al. 1996; Ho and Glimcher 2002).

The two main functional classes of T helper lymphocyte are Th1 and Th2 (Mosmann et al. 1986). IL-12, IL-23 and IL-27 are essential cytokines involved in polarizing T-helper cells towards a Th1 phenotype. Th1 cells secrete pro-inflammatory cytokines such as IFN-Ɣ, TNF-α, IL-2, IL-6 and IL-12 involved in immune response against bacterial and viral infections. Th2 cells secrete anti-inflammatory cytokines like IL-4, IL-5, IL-10, IL-13 and TGF-β (Murphy and Reiner 2002). IL-4 is a vital cytokine for the polarisation of T-lymphocytes towards Th2 phenotype. Furthermore, IL-4, IL-5 and IL-13 support B-lymphocyte proliferation, class switching and differentiation to effector cells. In summary, the differentiation of $\alpha\beta^+$ $CD4^+$ is regulated by T-cell receptor engagement. A number of cytokines and costimulatory signals as illustrated in figure 1.2.

More recently, two new T-lymphocyte populations, Th17 and Th22 characterized by IL-17 and IL-22 production, have been defined (Harrington et al. 2005; Eyerich et al. 2009). Both cytokines are involved in immune-mediated cutaneous reactions such as atopic dermatitis, allergic contact dermatitis and psoriasis (Eyerich et al. 2010; Cavani et al. 2012). IL-17 possesses strong pro-inflammatory activity, especially in the presence of INF-Ɣ. This results in T-cell-keratinocyte adhesion and T-cell-mediated cytotoxicity. On the other hand IL-22 plays a critical protective role, inducing keratinocyte proliferation and migration (Eyerich et al. 2009; Eyerich et al. 2010).

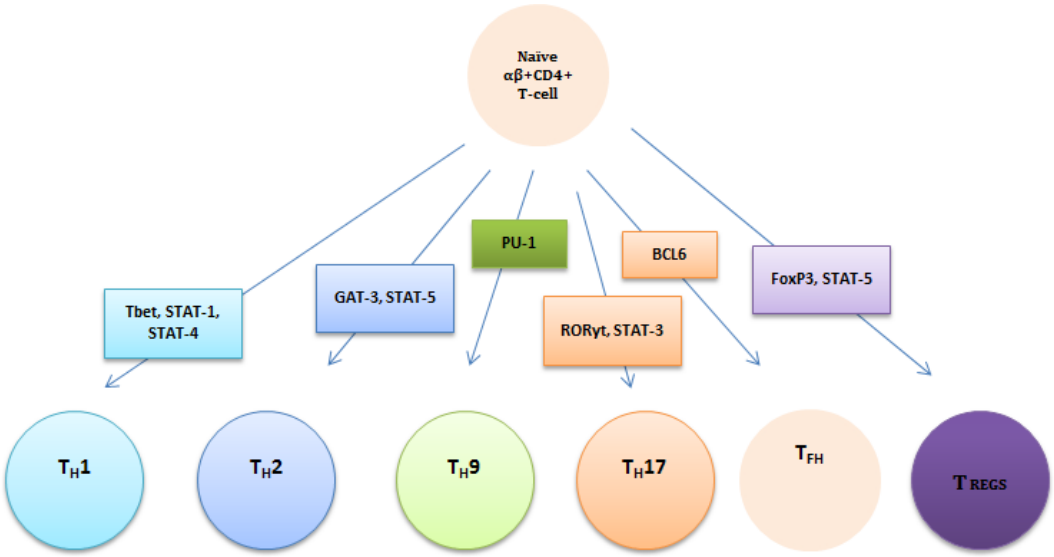


Figure 1.2-Schematic of T-cell development from naïve CD4+ T-cell. Plasticity of T-cell polarisation showing transcription factors involved. Tbet (T-box transcription factor), STAT (signal transducer and activator of transcription), GAT-3 (Anti-GABA Transporter-3), PU-1(macrophage transcription factor), RORYt (retinoic acid receptor-related orphan nuclear receptor gamma), BCL6 (B-cell lymphoma 6), FoxP3 (forkhead box P3), T_H (T helper), FH (follicular helper T cells), T_{REGS} (Regulatory T-cells)

Cytotoxic T-lymphocytes are involved in target cell destruction via a number of effector molecules resulting in necrotic cell death (Barry and Bleackley 2002). Perforin, FAS (CD95), granzyme-B and granulysin are the main death pathways for T-lymphocyte toxicity (Henkart 1985; Ostergaard et al. 1987; Helgason et al. 1992; Rouvier et al. 1993; Barry and Bleackley 2002; Chung et al. 2008). Upon antigen stimulation, cytotoxic T-lymphocytes secrete perforin which forms pores on the membrane of target cells. Perforin released from the granules of cytotoxic T-lymphocytes binds to granzymes and proteoglycans, forming a complex on the target cell membrane followed by enhanced entry of granzymes (usually A and B) and subsequent cytotoxicity. Furthermore, cytotoxic T-lymphocytes express the trimeric membrane protein, Fas ligand, which binds to

Fas receptors found on target cells resulting in the activation of caspase-8 and subsequent cytotoxicity. Like perforin, granulysin is secreted from granules of cytotoxic T-lymphocytes following antigenic stimulation. Granulysin disrupts lipids in the cell membrane by activating lipid-degrading enzymes resulting in cell death (Vaccaro et al. 1997; Ernst et al. 2000; Kaspar et al. 2001).

Regulatory T-lymphocytes (Tregs) are a heterogeneous group of $Foxp3^{+}CD25^{+}CD4^{+}$ lymphocytes that act to supress the immune system after an immune response. There are two distinct categories of regulatory T-lymphocytes namely, natural Tregs and inducible Treg. Tregs suppress the response of T-lymphocytes either through the secretion of immunosuppressive cytokines (IL-10, TGF-β) or via a CTLA-4 dependent pathway (Wing et al. 2008; Sakaguchi et al. 2009). Tregs constitute only a small fraction of CD4+ T-cells and express a diverse repertoire of αβTCR compared with conventional CD4+ T-cells (Pacholczyk and Kern 2008). However, similarities exist in the usage of TCR variable region segments Vβ in both humans and mice (Kasow et al. 2004; Fujishima et al. 2005).

1.2.2.1.2 B-lymphocytes

B-lymphocytes ($CD19^{+}$) are the only immune cells capable of antibody production following antigen recognition, thus conferring humoral immunity. B-lymphocytes express specialized cell surface proteins referred to as immunoglobulins which interact with antigens. This results in proliferation and differentiation into plasma and memory B-lymphocytes. Antibodies produced by plasma cells can neutralize viral material, fix complement and enhance phagocytosis through antigen opsonisation. Memory B-lymphocytes express high affinity IgA or IgG molecules and persist in the systemic circulation in a

resting stage but respond rapidly on re-encounter with specific antigen. Drug-specific IgE antibodies mediate immediate hypersensitivity reactions caused by the β-lactam antibiotics (Torres et al. 2003). IgG is the major antibody isotype found in blood and extracellular fluid and plays an important role in the control infection by binding to different pathogens. They have been detected in patients with delayed drug hypersensitivity reactions (Kiefel et al. 1987; Clarke et al. 1991) however, the role of IgG in the disease pathogenesis is still not clear.

1.3 Mechanisms of drug specific T- lymphocyte activation

Differentiating between the terms hapten, antigen and immunogen is important in order to delineate the molecular mechanism and immunological basis of drug-specific T-lymphocyte activation (Naisbitt et al. 2001; Uetrecht and Naisbitt 2013). A hapten can be defined as any low molecular weight compound capable of covalent and irreversible modification of macromolecules like proteins. A molecule that binds with high affinity to immunological receptors is referred to as an antigen. Finally, an immunogen is a substance that triggers an immune response.

Although DCs are the most prominent APCs, B-lymphocytes and macrophages are also involved in antigen presentation. T-lymphocytes inspect antigens presented on MHC-peptide complexes on APCs using their membrane borne TCRs.

The activation of naïve T-lymphocyte is dependent on at least two out of three signals from an APC namely, signals 1, 2 and 3. Signal 1 is derived from the interaction between the TCR with an MHC-peptide complex. Complete activation of a T-lymphocyte requires a co-stimulatory signal (signal 2). Co-

stimulation is regulated by a complex wave of receptor/ligand interaction. For example, the cell surface expression of CD80 and CD86 (CD28 ligands) on APCs is controlled via trans-endocytosis by cytotoxic T lymphocyte antigen 4 (CTLA4). Although CD28 was the first co-stimulatory molecules to be identified on T-cells, newer molecules involved in co-stimulation have been identified and characterised (Chen and Flies 2013). These molecules are important for T-cell activation but also in T-cell differentiation, survival and effector function. A third signal (signal 3) derived from cytokines secreted by APCs drives $CD4^+$ T helper cell and $CD8^+$ lymphocyte differentiation into effector cells. Signal 3 determines the nature of an immune response as it conveys critical information on pathogen and infected tissues to T-lymphocytes.

A number of structural and functional similarities exist between MHC class I molecule and MHC class II molecules, but they differ markedly in the origin and nature of the peptide they present to different subsets of T-lymphocytes (Berzofsky 1988; Deng et al. 2010). MHC class I molecules are expressed on the surface of all nucleated cells and display peptides derived from intracellular proteins to $CD8^+$ T-lymphocytes. This is referred to as cytosolic or endogenous processing (Neefjes et al. 2011). On the other hand, MHC class II molecules are stably expressed on the surface of APCs but are up-regulated in other human cells following inflammation or activation. MHC class II presents peptides derived from extracellular proteins (endocytic or exogenous processing) to $CD4^+$ lymphocytes (Romieu-Mourez et al. 2007; Vyas et al. 2008; Neefjes et al. 2011). MHC class I molecules are sub-divided into HLA-A, -B and C while the different sub-classes of MHC class II molecules are HLA-DP,-DQ and -DR which are differentially expressed on B-lymphocytes, activated monocytes, EBV-

transformed B-cell lines, Kupffer cells, Langerhan cells and dendritic cells (Radka et al. 1986; Alcaide-Loridan et al. 1999).

Exceptions to the classical MHC restricted presentation of peptides exist. Peptides derived from extracellular proteins are presented on MHC class I molecules by a specialized subset of dendritic cells through adaptations in their endocytic and phagocytic pathways. This is referred to as cross presentation and vital for the induction of $CD8^+$ T-lymphocyte responses by extracellular antigens (Jung et al. 2002; Joffre et al. 2012). Furthermore, peptides derived from cytosolic proteins have been shown to be presented on MHC class II molecules through autophagy (Crotzer and Blum 2010; Munz 2012).

Cytosolic proteins undergo proteasomal degradation to form peptide fragments which are translocated to the endoplasmic reticulum (ER) by the Transporter associated with antigen presentation (TAP). TAP binds to freshly synthesised MHC class I molecules and then conveyed through the Golgi apparatus to the cell membrane for $CD8^+$ T-lymphocyte recognition (Neefjes et al. 2011). Extracellular proteins engulfed through phagocytosis are digested by proteasomal enzymes contained in lysosomes. Processed peptides are subsequently presented on MHC class II molecules for $CD4^+$ lymphocyte recognition. Figure 1.3 illustrates cytosolic and extracellular antigen processing.

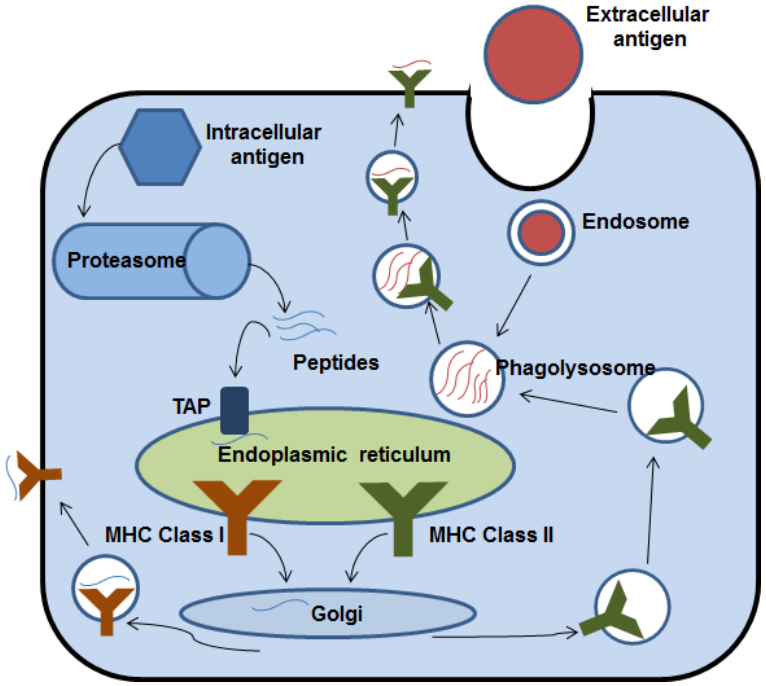


Figure 1.3- Antigen processing and presentation. Extracellular antigens are taken up by phagocytosis and processed by enzymes in the endosome. MHC class II molecules are transported from the endoplasmic reticulum and Golgi in vesicles to the phagolysosome where peptide loading occurs. The MHC bound peptide is then transported to the cell surface where the antigen is displayed to T-lymphocytes. Intracellular antigens degraded in the proteasome are transported to the endoplasmic reticulum by the transporter of antigen processing (TAP) and bind to MHC class I molecules. The MHC-bound peptide is then trafficked to the Golgi where it is loaded into a vesicle and transported to the cell surface for antigen display.

Peptides are known to interact within certain groves on MHC molecules through non-covalent but sequence dependent interaction, mainly hydrogen bonds and Van der Waals forces (Jensen 2007). Specific amino acid residues known as anchor residues facilitate the peptide-MHC interaction. MHC class I accommodates only peptides of between 8-9 amino acid subunits. This is because of non-covalent interactions between both ends of the peptide binding groove and free N and C termini of peptide (Matsumura et al. 1992). The amino acid residue at position 5 is critical for the TCR/peptide interaction (Speir et al. 2001; Rudolph and Wilson 2002). On the other hand, MHC class II interacts with peptide lengths between 12-25 amino acid subunits, with less dependence on

specific amino acid residues for TCR and MHC-peptide complex interaction (Rammensee 1995). The antigen binding grove of MHC class II molecules are open at both ends; hence the interaction with longer peptides when compared to the binding grove of MHC class I molecules (Jensen 2007; Neefjes and Ovaa 2013).

The outcome of the MHC-peptide complex interaction with the TCR is a complex chain of downstream signalling events leading to T-lymphocyte proliferation, differentiation, and secretion of cytokines and cytotoxic molecules. This interaction between APCs and T-lymphocytes occurs through several pairs of accessory molecules (CD4 or CD8 coreceptors) resulting in aggregation of TCRs on the membrane surface and subsequent activation of T-lymphocytes (Choudhuri et al. 2005). The signalling cascade that precedes T-lymphocyte activation begins with the phosphorylation of tyrosine proteins by the Src kinases and Lck (Nel 2002). Phosphorylation of TCRs by Lck creates a binding site for the recruitment of another protein tyrosine kinase (PTK) called ZAP-70 (zeta chain associated protein of 70kDa) (Smith-Garvin et al. 2009). ZAP-70 then phosphorylates two critical adaptor proteins namely LAT (linker for activation of T-lymphocytes) and SLP76 (SH2-domain containing leukocyte protein of 76kDa), triggering the release of Ca^{2+} and diacylglycerol (DAG) and subsequent activation of T-lymphocytes. Several concepts have been put forward to explain how drugs activate T-lymphocytes. The details of these concepts are discussed in the following sections.

1.3.1. Hapten hypothesis

The basis of the hapten hypothesis dates back to early research carried out by Landsteiner and Jacobs in 1935. For decades, this was the only explanation for the immunogenicity that resulted from small molecules. They investigated the mechanism by which small chemical molecules activated T-lymphocytes (Landsteiner and Jacobs 1935). Their work involved sensitising guinea pigs to the low molecular weight, chemically reactive compound, dinitrochlorobenezene (DNCB). The authors reported that the primary response observed with DNCB was due to the modification of nucleophilic residues on proteins and associated with their chemical reactivity *in vitro* (Landsteiner and Jacobs 1935; Landsteiner and Jacobs 1936). They therefore hypothesised that the mechanism of sensitization may involve protein haptenation by DNCB.

A more recent study used DNCB as a model chemical to investigate the metabolic fate of dinitrophenyl hapten conjugated albumin (Kitteringham et al. 1985). The authors reported the clearance of DNP-modified albumin depended on the extent of haptenation. Furthermore, the role of DNCB responsive T-cells have been isolated from hypersensitive individuals and shown to be implicated in the pathomechanism of this reaction (Pickard et al. 2007).

Therefore, low molecular weight compounds such as drugs and their metabolites might also act as haptens and are capable of activating an immune response when bound covalently to proteins (Landsteiner and Jacobs 1935; Speirs 1971; Park et al. 1987; Chipinda et al. 2011). Prohaptens are inert drugs that become chemically reactive following metabolism (Brander et al. 1995; Naisbitt et al. 2001; Pichler 2003). Selective binding to lysine or cysteine amino

acid residues have been reported with piperacillin and SMX respectively (Padovan et al. 1996; Naisbitt et al. 2001; Callan et al. 2009; El-Ghaiesh et al. 2012; Monshi et al. 2013). The interaction between nucleophilic sites on proteins and electrophilic sites on small chemical molecules represents the basis of the hapten hypothesis (Divkovic et al. 2005; Chipinda et al. 2010). Figure 1.4 is a schematic representation of the Hapten hypothesis. Chemically reactive molecules become immunogenic by either binding to extracellular proteins that are engulfed by APCs, processed and presented to T-cells or direct modification of MHC-peptides (figure 1.4).

Nakayama and his colleagues demonstrated a correlation between the ability of a drug to generate reactive drug metabolites and idiosyncratic drug reactions (Nakayama et al. 2009). Paradoxically, Ximelagatran, a drug developed as a replacement for the anticoagulant warfarin presented with high risks of idiosyncratic drug reactions without generating reactive metabolites (Hirsh et al. 2007; Uetrecht 2008).

Glutathione is highly concentrated in the liver but differentially expressed in almost all human cells (Perquin et al. 2000; Coles et al. 2001). It represents the major anti-oxidant pathway critical in the body's detoxification process of drugs and xenobiotics. In the presence of high concentrations of reactive metabolite, the glutathione detoxification pathway is overwhelmed resulting in: (a) protein haptenation and subsequent stimulation of the adaptive immune system and (b) activation of the innate immune system following stimulation of stress-related signalling pathways (Uetrecht and Naisbitt 2013).

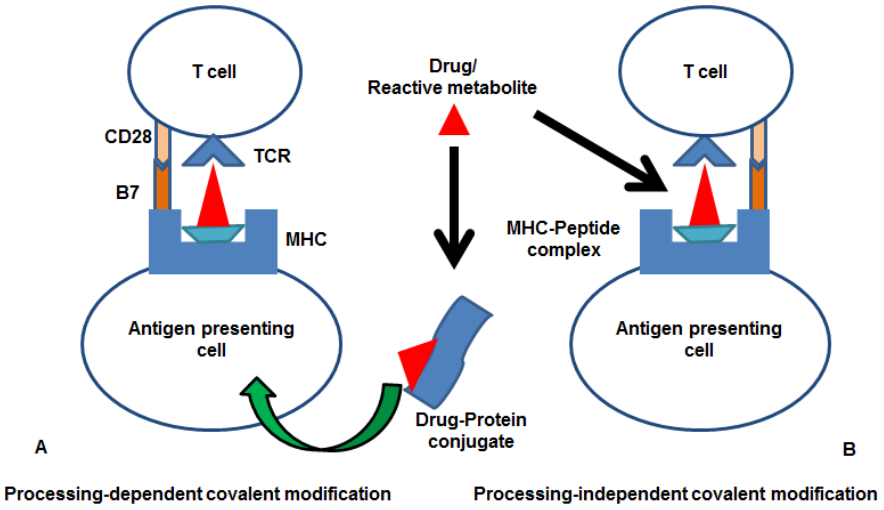


Figure 1.4 Hapten hypothesis. Parent drugs or their reactive metabolites covalently modify macromolecules like proteins. **(A)** Drug-modified proteins are engulfed and then processed by antigen presenting cells and presented in an MHC restricted fashion to T-lymphocytes. **(B)** Parent drugs or reactive metabolites directly modify the MHC–peptide complex and are presented to T-cells.

1.3.2. Pharmacological interaction of drugs with immune receptors (PI concept)

The discovery that some drug-specific T-lymphocytes could be activated by chemically inert drugs in a metabolism- and processing-independent, but MHC-restricted pattern, is the basis for the p-i concept (Schnyder et al. 1997; Horton et al. 1998; Zanni et al. 1998; Pichler 2002). Examples of drugs known to activate T-lymphocytes via a p-i mechanism include carbamazepine (Naisbitt et al. 2003; Wu et al. 2006), lidocaine (Naisbitt et al. 2003), lamotrigine (Naisbitt et al. 2003), mepivacaine (Zanni et al. 1999), norfloxacin (Schmid et al. 2006; Schmid et al. 2006), and SMX (Schnyder et al. 1997; Depta et al. 2004). This observed pattern of T-lymphocyte activation is inconsistent with the hapten hypothesis, where proteins are covalently modified by chemically reactive molecules (figure 1.4). This is largely because T-cells are activated rapidly (within seconds), a time incompatible with a processing dependent pathway

(Schnyder et al. 1997; Zanni et al. 1998; Schnyder et al. 2000). The critical question yet to be addressed is which of these entities (parent drug or reactive metabolites) activate T-cells during a hypersensitivity reaction and which the cross reactive antigen is.

There are three main pieces of experimental evidence to support a p-i mechanism of T-lymphocyte activation. (1) Glutaraldehyde-fixed APCs incapable of antigen processing have been shown to induce T-lymphocyte proliferation in response to carbamazepine (Wu et al. 2006), SMX (Schnyder et al. 1997), lamotrigine (Naisbitt et al. 2003), ciprofloxacin (Schmid et al. 2006), moxifloxacin (Schmid et al. 2006), and norfloxacin (Schmid et al. 2006). (2) The release of intracellular Ca^{2+} in response to drug stimulation and TCR down regulation are cellular processes that occur rapidly, excluding any possibility of antigen processing (Zanni et al. 1998; Depta et al. 2004). (3) Pulse-chase experiments with 'culprit drug' suggest a non-covalent interaction of drug with the MHC-peptide complex and TCR as a brief washing with medium abolished T-lymphocyte proliferation (Schnyder et al. 1997; Schnyder et al. 2000). In conclusion, these data provides strong evidence to suggest that drugs activate T-cells directly in a mechanism different from the hapten/pro-hapten hypothesis. Figure 1.5 illustrates the antigen recognition by drug-specific T-lymphocytes according to the p-i mechanism.

Importantly, SMX hypersensitivity is the only example where systemic reactive metabolites are available for functional studies to compare the hapten and the P-i mechanisms of T-cell activation. Interestingly, 100% of T-cells isolated from blood and skin of SMX-hypersensitive patients are activated by both SMX and SMX metabolites (Schnyder et al. 2000; Burkhart et al. 2001; Nassif et al. 2002;

Castrejon et al. 2010). This suggests that a combination of the hapten and the Pi mechanisms are important in pathophysiological mechanism of SMX hypersensitivity.

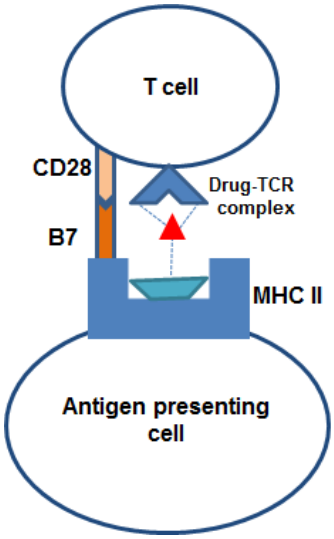


Figure 1.5 T-lymphocyte recognition of drug antigen by pharmacological interaction with immune receptors. Drug or their reactive metabolites bind non-covalently to either TCR or MHC-peptide complex or both to. Dash line represents non-covalent interactions.

1.3.3 Altered self-peptide repertoire model

The pathogenesis of certain drug hypersensitivity reactions have been strongly linked to particular human leukocyte alleles, HLA (Mallal et al. 2002; Chessman et al. 2008; Mallal et al. 2008; Bharadwaj et al. 2012; Yun et al. 2012). HLAs are highly heterogeneous proteins encoded within the MHC gene, located on chromosome 6. They initiate immune reactions through the presentation of peptides to TCR (McCluskey and Peh 1999; Illing et al. 2012; Pavlos et al. 2012). Figure 1.6 illustrates the altered peptide repertoire hypothesis.

Abacavir hypersensitivity syndrome is strongly associated with HLA-B*57:01 and has been used to describe the altered self-peptide repertoire hypothesis (Mallal et al. 2002; Martin et al. 2004; Mallal et al. 2008). It proposes that drug

molecules occupying particular sites in the antigen-binding cleft cause an alteration in the repertoire of self-peptides that interact with HLA-B*57:01 and are subsequently presented to TCRs (Chessman et al. 2008; Bharadwaj et al. 2012).

Abacavir interacts in a specific but non-covalent manner with the F anchor pocket of HLA-B*57:01, changing the configuration of the antigen-binding cleft (Illing et al. 2012; Norcross et al. 2012; Ostrov et al. 2012). The altered repertoire of self-peptides displayed on the surface of APCs is thought to activate abacavir specific cytotoxic $CD8^+$ T-lymphocytes; the cells responsible for the clinical manifestation of abacavir hypersensitivity syndrome (AHS).

Pre-prescription screening of patients for the risk allele, HLA-B*57:01 has become a recommended guideline before initiating abacavir therapy in HIV patients (Hughes et al. 2004; Rodriguez-Novoa et al. 2007). On the other hand, the low positive predictive value (PPV) between HLA-B*57:01 allele and flucloxacillin, which is associated with flucloxacillin-induced liver injury, eliminates any need for pre-prescription screening before commencing flucloxacillin therapy (Yun et al. 2012).

Stevens-Johnson syndrome and toxic epidermal necrolysis are serious cutaneous clinical manifestations of the anticonvulsant carbamazepine. Reactions in the Han Chinese are strongly associated with HLA-B15:02 and has been hypothesised that drug binding to the HLA molecule alters peptide binding similar to abacavir. However, this is still to be proven (Chung et al. 2004; Hung et al. 2006). Finally, severe cutaneous adverse reactions reported following the administration of the antigout drug, allopurinol, are associated with HLA-B*58:01 in Han Chinese and in Europeans (Hung et al. 2005; Lonjou et al. 2008).

Drug-specific T-cells in patients with allopurinol hypersensitivity have recently been shown to be preferentially activated with oxipurinol, the principal metabolite (Yun et al. 2013; Yun et al. 2014), but the nature of the drug interaction with immunological receptors is yet to be defined.

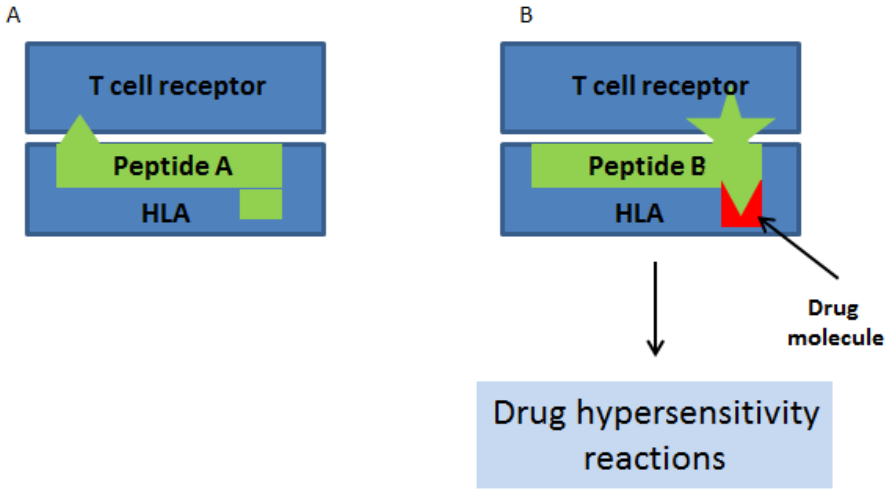


Figure 1.6-Altered peptide repertoire model. (A) Normal interaction of peptide A with HLA molecule and subsequent interaction with TCR. **(B)** The presence of a drug molecule alters the repertoire of HLA ligand (peptide B) presented to TCR resulting in hypersensitivity drug reactions.

1.3.4 Danger hypothesis

The danger model was proposed by Matzinger in 1994 and states that in the presence of danger signals from cell damage, the immune system is activated, but tolerance is induced in its absence (Matzinger 1994; Schwartz 1997; Anderson and Matzinger 2000; Pirmohamed et al. 2002; Schwartz 2003). Thus, danger signals complement the primary antigenic signal in propagating an immune response (Mueller et al. 1989; Seguin and Uetrecht 2003). According to Curtsinger and his colleagues, three signals are vital to bring about a complete immune response; at least two signals are required for immune activation. (Curtsinger et al. 1999). The interaction between the MHC-peptide complex and

the TCR generates signal 1 (Danese et al. 2004). The interaction between the various costimulatory molecules on APCs (CD40, CD80 and CD86) and T-lymphocytes (CD28, CD40L) delivers signal 2. Pro-inflammatory cytokines like interferon gamma, interleukin-2 and tumour necrosis factor alpha up-regulate co-stimulatory molecules on antigen presenting cells. A wave of co-stimulatory/inhibitory receptors expressed by T-cells and APCs have been extensively reviewed (Chen and Flies 2013). Signal 2 is essential for T-lymphocyte clonal expansion, cytokine secretion and effector functions. CD28 molecules expressed on the surface of T-lymphocytes interact with CD80 and CD86 on the surface of APCs to promote T-lymphocyte proliferation, differentiation and survival (Hancock et al. 1996; Van Gool et al. 1996). Signal 2 is modulated through exogenous PAMPs (eg. LPS, peptidoglycan, viral RNA) and endogenous DAMPs released from damaged/dead cells (eg. heat shock proteins, HMGB1) as illustrated in figure 1.7.

Nitroso-SMX, abacavir and amoxicillin have been reported to provide danger signals for immune activation (Rodriguez-Pena et al. 2006; Martin et al. 2007; Sanderson et al. 2007). However, danger signals may result from chemicals exposure, physical trauma and /or infections. The role of viruses (human herpesvirus 6, human immunodeficiency virus and Epstein barr virus) in drug-induced hypersensitivity syndrome has been extensively researched and sometimes complicates diagnosis (Descamps et al. 1997; Descamps et al. 2003; Phillips and Mallal 2007).

Finally, Signal 3 is derived mainly from polarising cytokines that result in either Th1, Th2, Th17 or Th22 immune response (Pirmohamed et al. 2002). Chemically reactive drug metabolites have been reported to serve as an antigen (signal 1) but also induce cell damage resulting in the generation of signal 2 and 3 required for an immune response (Park et al. 2001; Li and Uetrecht 2010).

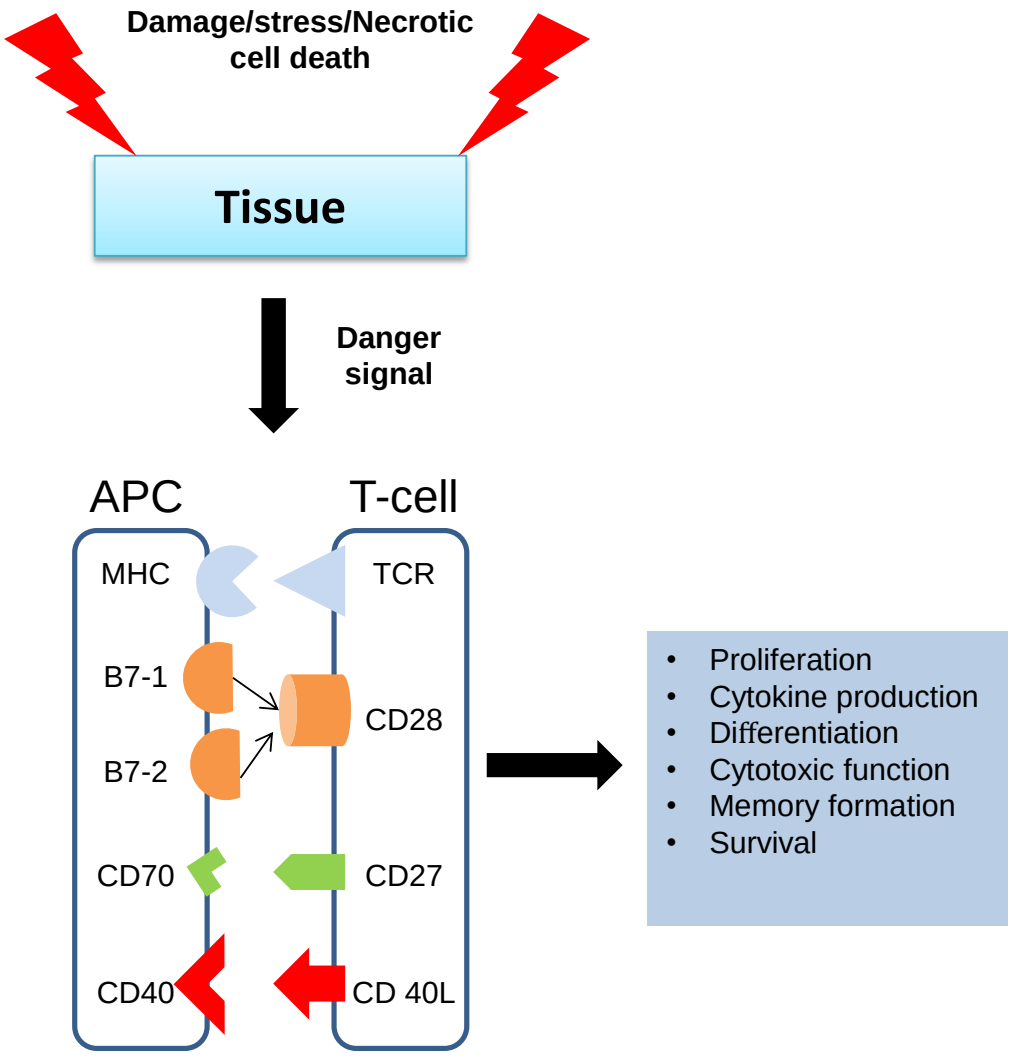


Figure 1.7-T-cell activation. Schematic representation of antigenic signal, danger signal and co-stimulatory signals critical for T-cell activation.

1.4 Xenobiotic and drug metabolism

The terms xenobiotic metabolism and drug metabolism are often used interchangeably. Drug metabolism is defined as the biomedical modification of xenobiotics by specialised enzyme systems. The term “drug metabolism” was coined in the 1950s to replace drug detoxification as not all drugs are toxic and not all metabolites are less toxic or non-toxic (Ala, 2005). Generally, the level of exposure of drugs to human tissue is controlled by a combination of metabolic, biliary and renal clearance. Highly lipophilic, less water soluble drugs are converted into more soluble metabolites which are easily excreted.

Drug metabolism plays an important role in the initiation and propagation of drug hypersensitivity through the generation of neoantigens that are recognised by the cellular and humoral immune systems (Lavergne et al. 2008). Although the majority of drug biotransformation occurs in the liver, there is overwhelming evidence to suggest that localised drug metabolism by immune cells is critical for cutaneous adverse drug reactions (Ju and Uetrecht 1999; Baron et al. 2001; Saeki et al. 2002; Oesch et al. 2007). In the absence of underlying pathologies or risk factors such as age, disease status, and enzyme induction, a fine balance occurs between bioactivation and bioinactivation pathways (Pirmohamed et al. 1998).

While most parent drugs administered form stable metabolites which are excreted safely, a number of drugs exert direct toxicity on cells and tissues (figure 1.8). In most cases, reactive metabolites generated from drugs administered at therapeutic doses are quickly saturated by cellular glutathione detoxification pathways but a few exceptions have been documented

(Pirmohamed et al. 1996). Drug overdose can result in glutathione depletion and saturation of the cellular detoxification pathways. High levels of reactive metabolites are formed resulting in irreversible covalent protein modification, with serious and sometimes fatal pathologic outcomes (Nelson 1990; Bray 1993). Glutathione depletion also occurs in certain disease states (HIV, Cystic fibrosis), and in alcoholic patients leading to an increase in cellular levels of reactive metabolites (Staal et al. 1992; Roum et al. 1993; Leach et al. 1998). These patients are particularly susceptible to the development of drug hypersensitivity. Interestingly, the pharmaceutical industry has devoted significant resources to delineate whether reactive metabolites are formed and the role of reactive metabolites in drug toxicity and hypersensitivity reactions (Baillie et al. 2002). Despite this, our understanding of the relationship between drug exposure, metabolite formation and the development of serious ADRs is far from complete.

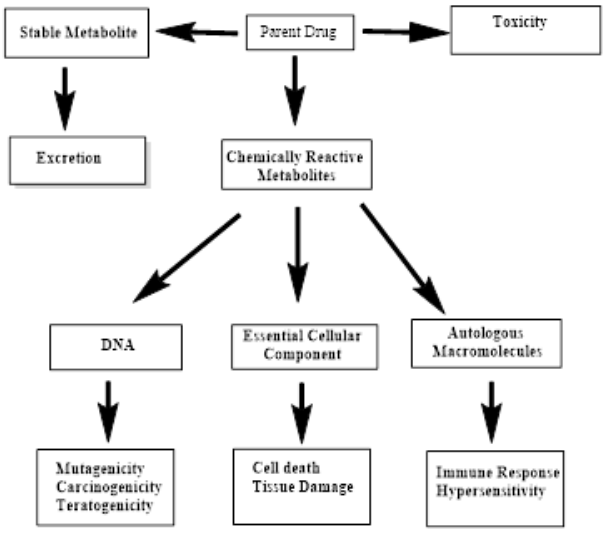


Figure 1.8-Typical metabolism pathway of most drugs in humans (adapted from Park et al., Chem Biol Interact. **192(1-2)**:30-6, 2011).

1.4.1 Drug metabolism enzymes

Drug metabolism enzyme expression and substrate specificity are critical factors in selective tissue toxicity associated with drug exposure. Over 95% of drug metabolism occurs in the liver. However, extra-hepatic tissues also express different active drug metabolism enzymes (Kapitulnik and Strobel 1999; Pirmohamed et al. 2004; Swanson 2004; Uetrecht and Naisbitt 2013). Immune cells such as polymorphonuclear leukocytes have been reported to express low levels of cytochrome P450 enzyme and high levels of myeloperoxidase (Uetrecht et al. 1988; Maggs et al. 1995).

Drug metabolism enzymes are broadly classified as microsomal and non-microsomal. Microsomal enzymes are found in the smooth endoplasmic reticulum of the liver, lungs, kidneys and intestinal mucosa. They catalyse a vast number of drug biotransformation reactions. Microsomal enzymes can be induced by a number of drugs like alcohol, rifampicin, phenytoin, carbamazepine, phenobarbitone and many others (Yaffe et al. 1966; Callaghan et al. 1977; Miguet et al. 1977; Mapelli et al. 1983). Examples of microsomal enzymes include the Cytochrome P450 super family (CYP450s), flavine containing mono-oxygenases (FMOs), epoxide hydrolase (EHs) and uridine diphospho-glucuronosyltransferase (UGT). Non microsomal enzymes on the other hand are present in the cytoplasm and mitochondria of cells as well as in the plasma. They are non-specific, non-inducible and catalyse fewer oxidative, reductive and hydrolytic reactions. They are involved in a number of conjugation reactions (glucouronidation being an exception). Examples of non-microsomal enzymes include amidases and esterases. Drug metabolising

enzymes especially Cytochrome P450's have been functionally characterised in almost every tissue type in the human body.

1.4.1.1 Cytochrome P450 super family

The Cytochrome P450 superfamily of enzymes are membrane-bound hemoproteins found in the smooth endoplasmic reticulum and are abundant in the liver and other extrahepatic tissues (Pirmohamed et al. 1996). They can also be found in the inner membrane of the mitochondria and account for about 75% of drug metabolism within the human body (Guengerich 2008; Berka et al. 2011). About 60 members of the CYP family have been characterized but only 10 members contribute significantly to biosynthetic pathways (hormones and structural components) and drug metabolism. Oxidation is the most common metabolic reaction catalysed by the CYP P450 family of enzymes. Cytochrome P450-mediated drug metabolism presents practical applications in many aspects of drug design and development but also in drug therapy (Werck-Reichhart and Feyereisen 2000). Variations in certain human P450 genes like CYP2D6 have profound implications in the metabolism of antipsychotics and antidepressants (Rau et al. 2004; Ingelman-Sundberg 2005). These enzymes are structurally similar and have a common general biochemical mechanism of action which is highly dependent on electron transfer from redox partners (NADPH).

The nomenclature of the different CYPs is based on the amino acid sequence similarity of various gene products and is undertaken by a special nomenclature committee as represented in figure 1.9. CYP1 (1A &1B), CYP2 (2A, 2B, 2C, 2D & 2E) and CYP3 (3A) constitute the major CYP family with eight sub-families all together that account for the metabolic elimination of most drugs and

xenobiotics in humans (Rendic and DiCarlo 1997; Guengerich 2003; Williams et al. 2004). About 90% of drugs administered are metabolised by CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 (Slaughter and Edwards 1995; Wilkinson 2005). CYP3A4 is the most abundant member in human liver and responsible for the biotransformation of many classes of drug (Warrington et al. 2000; Anzenbacher and Anzenbacherova 2001). Each sub-family consists of only one member except CYP2C which has three members. Figure 1.10 shows the members of the Cytochrome P450 super family. The cytochrome P450 family of enzymes are involved in the metabolism of a structurally diverse group of drugs as displayed in table 1.7.

The induction or inhibition of CYP450 enzymes can cause alterations in the normal pharmacokinetics of a given drug and may result in life-threatening ADRs. Drug-drug interactions are sometimes complex and are implicated in about 20% of all ADRs (Levy et al. 1980).

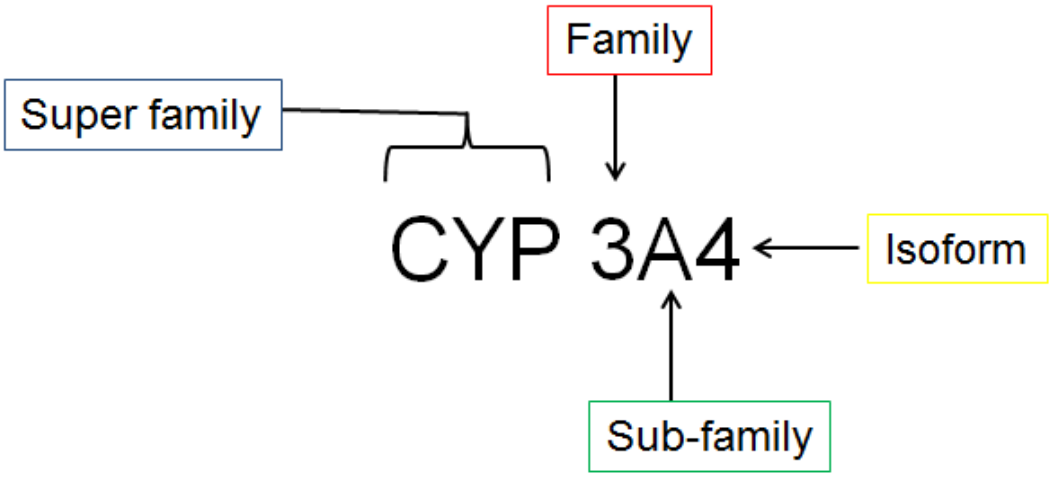


Figure 1.9–General nomenclature of the Cytochrome P450 superfamily of enzymes.

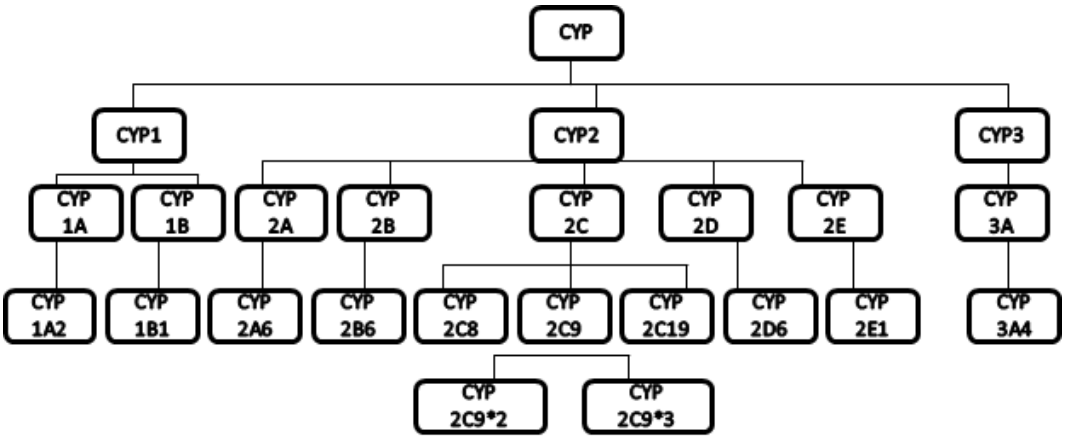


Figure 1.10-Family members of the Cytochrome P450 super family involved in drug metabolism (Adapted from Nassar, A.F., et al, drug metabolism hand book, 2009).

Table 1.7- Cytochrome P450 enzymes and their selective substrate (Adapted from Nassar, A.F. et al., drug metabolism handbook, 2009)

Enzyme	Substrate
CYP1A2	Amitriptyline, Betaxolol , Verapamil , (R) - Warfarin, Haloperidol, Imipramine, Propranolol, Olanzapine, Clozapine.
CYP2A6	Nicotine, Betadine, Coumarin.
CYP2C9	Piroxicam, Naproxen, Fluoxetine, Ibuprofen, Tolbutamide, Phenytoin, (S)- Warfarin, Lorsatan, Diclophenac
CYP2C19	Omeprazole, Imipramine, Diazepam, Amitriptyline.
CYP2D6	Amitriptyline, Betaxolol, Codeine, Clozapine, Fluoxetine, Haloperidol, Imipramine, Methadone, Metochlopramide , Propranolol, Sertraline, Timolol
CYP2E1	Acetaminophen, Caffine, Dextromethorphan, Ethanol, Theophylline
CYP3A4/5	Amiodarone, Ami triptyline, Astemizole, Caffeine, Carbamazepine, Codeine, Cyclosporine, Dexamethasone, Felodipine, Erythromycin, Clarithromycin, Lansoprazole, Linocaine, Imipramine, Omeprazole, Ophenadrine, Rifampicin, Paroxetine, Progesterone, Sildenafil,

1.4.1.2 Flavine-containing monooxygenases (FMOs)

FMOs are microsomal phase I drug metabolism enzymes involved in the NADPH-dependent biotransformation (N-and S-oxidation reactions) of a number of drugs. They are also capable of generating a number of reactive intermediates (Pritsos et al. 1985; Cashman 1995). FMOs and the CYPs enzymes are co-localized in the endoplasmic reticulum. The five functionally active FMOs characterized in humans include: FMO1, FMO2, FMO3, FMO4, and FMO5. Table 1.8 summarises expression and substrate selectivity of the FMOs. FMOs have

been implicated in hepatotoxicity resulting from N-acetylation and sulfoxidation of ketoconazole and thioureas respectively (Rodriguez and Miranda 2000; Rodriguez and Buckholz 2003; Henderson et al. 2004). FMO1 and FMO3 are involved in the metabolism of SMX and dapsone in normal human epidermal keratinocytes (NHEKs) and KG-1 (dendritic-like) cells respectively (Vyas et al. 2006; Roychowdhury et al. 2007). Both enzymes are also involved in the N-oxygenation of tamoxifen and other tertiary amines (Krueger et al. 2006).

Table 1.8 - Expression and substrate specificity of the major flavin-containing monooxygenase (FMOs)

FMO	Expression	Subsrates	References
FMO1	Fetal liver, Kidney, intestine	Xanomeline, methyl metabolite of disulfiram	Cashman and Zhang (2006)
FMO2	Lung	Thioureas, thioethers	Cashman et al. (2000)
FMO3	Liver	Trimethylamine, (S)-nicotine, clozapine, cimetidine, ranitidine	Cashman et al. (2000)
FMO4	Liver	No selective substrate identified	Cashman et al. (2000)
FMO5	Liver	Thioethers	Ohmi et al. (2003)

1.4.1.3 Peroxidases

Peroxidases represent an important class of heme-containing enzymes. They are expressed in high levels in polymorphonuclear granulocytes (PMN) and are involved in the bioactivation a large number of drugs in humans. They catalyse a number of reactions known to generate reactive drug metabolites resulting in agranulocytosis (Fischer et al. 1991; Besser et al. 2009). Myeloperoxidase (MPO), eosinophil peroxidase (EPO), and lactoperoxidase (LPO) are common examples of peroxidases involved in drug metabolism (Tafazoli and O'Brien

2005). Other peroxidases include uterine peroxidase, salivary peroxidase, thyroid peroxidase and prostaglandin H1/2 synthases (O'Brien 2000).

MPO is the most abundant and best characterised of the peroxidase family of enzymes. It is a lysosomal peroxidase primarily expressed in PMN; however, expression has also been noted in the liver, monocytes, macrophages, dendritic cells and mast cells (Pirmohamed et al. 1995; Green et al. 2004; Mannargudi et al. 2009; Kiorpelidou et al. 2012). MPO is involved in the metabolism of a wide range of drugs including: ticlopidine, clozapine, procainamide, clozapine, dapsone, chlorpromazine, lamotrigine, sulfonamides, 3-hydroxy carbamazepine, 4-hydroxy phenytoin, vesnarinone and 7-hydroxy fluperlapine (Liu and Uetrecht 2000; O'Brien 2000; Tafazoli and O'Brien 2005; Lu and Uetrecht 2007).

Upon neutrophil activation, MPO is released into phagocytic vacuoles alongside hydrogen peroxide, generated by the activity of NADPH oxidase and superoxide dismutase. Hydrogen peroxide combines with chloride ions in the MPO mediated synthesis of hypochlorous acid. Hypochlorous acid is known to directly oxidise many drugs but also cause direct tissue damage (Klebanoff 1999). The catalytic activity of MPO is summarized in the reaction cycle presented in figure 1.11. Compound I and compound II (active forms of the enzyme) are involved in the oxidation of a wide range of endogenous substrates and xenobiotic molecules (Tafazoli and O'Brien 2005; Winterbourn et al. 2006).

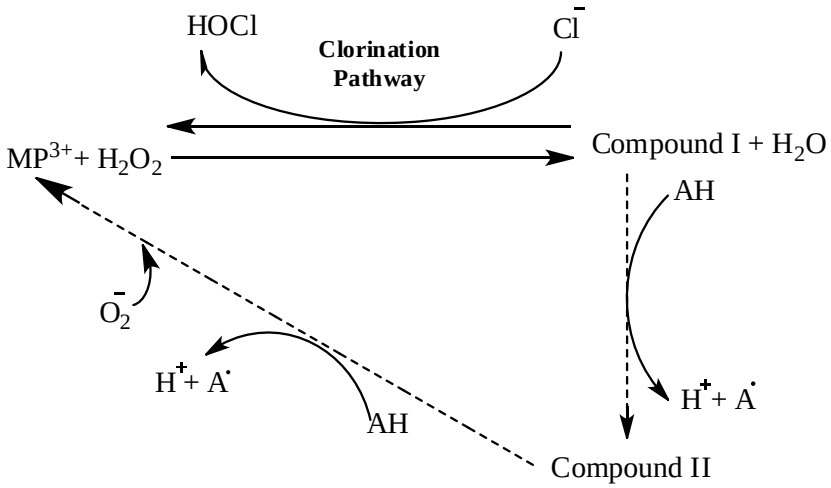


Figure 1.11 Redox transformations of myeloperoxidase. MP^{3+}, native MPO; AH, peroxidase substrate; A*, peroxidase substrate radical.

1.5 Drug Metabolism Pathways

R.T. Williams in 1947 was the first to describe and classify the pathways of drug metabolism into two groups, Phase I and Phase II reactions (Neuberger and Smith 1983). These reactions are catalysed by extrahepatic microsomal enzymes, hepatic microsomal enzymes, and hepatic non-microsomal enzymes (Kapitulnik and Strobel 1999).

1.5.1 Phase I drug metabolism reactions (functionalization)

Addition of reactive and more polar groups (functionalization) to substrates is a unique characteristic of Phase I metabolism enzyme. These functional groups include hydroxyl (-OH), amino ($-NH_2$), carboxyl (-COOH), and sulfhydryl (-SH) group. These groups are often the targets for phase II reactions. Oxidation of chemical moieties is the most important outcome of phase I metabolism; hydroxylation, reduction and hydrolysis occur to a lesser extent (Vonmoltke et al. 1994; Nemeroff et al. 1996; Stachulski and Lennard 2000). Paracetamol, phenothiazine and steroids are common examples of drugs that undergo phase I

metabolism. Sufficiently polar metabolites resulting from Phase I reactions are readily excreted after undergoing conjugation reaction with endogenous substrates. The resulting conjugates are highly polar and water soluble. Finally, Phase I metabolism enzymes especially members of the cytochrome P450 super family generate chemically reactive metabolites implicated in a number of idiosyncratic hypersensitivity drug reactions (Park et al. 1992; Pirmohamed et al. 1994; Pirmohamed et al. 1996).

1.5.2 Phase II drug metabolism reactions (Conjugation reactions)

A number of functional groups are introduced during Phase I biotransformation. These groups enhance the rate of Phase II conjugation. Phase II reactions consist of conjugation, methylation and acetylation reactions. In most cases, Phase I functionalization reactions normally precede conjugation reactions but a number of exceptions exist where parent drugs undergo direct conjugation, for example acetylation of sulfonamides (Someya et al. 1992; Kassahun et al. 1997).

Activated drug metabolites form conjugates with endogenous molecules such as glycine, glucuronic acid, glutathione, and sulfate to produce highly polar and less toxic adducts which are easily excreted from the body. Phase II reactions are catalysed by a range of substrate specific transferases. Glutathione S-transferases have been widely researched and are the most important enzymes involved in the detoxification of chemically reactive intermediates (Jakoby and Ziegler 1990; Liston et al. 2001; Homolya et al. 2003).

1.6 Reactive drug metabolites

Although drug metabolism serves as a major detoxification pathway which protects cells and tissues from the harmful effect of drugs, generation of drug reactive metabolites remain a major problem. Many drugs associated with a high incidence of idiosyncratic ADRs form reactive drug metabolites (see table 1.9) and bind covalently to cellular macromolecules (Spielberg et al. 1981; Shear et al. 1986; Liu et al. 1995; Ruscoe et al. 1995; Park et al. 2001; Stepan et al. 2011).

Table 1.9-Drug and reactive metabolites implicated in adverse drug reactions (adapted from Walgren et al., *Crit. Rev. Toxicol.,* **35**, 325-361 (2005)

Drug Name	Reactive Metabolite Formation
A. Drug withdrawn	
Benoxaprofen	Yes
Bromfenac	N/A
Iproniazid	Yes
Nefazodone	Yes
Tienilic acid	Yes
Troglitazone	Yes
B. Drug with a black box warning	
Acitretin	N/A
Bosentan	N/A
Dacarbazine	Yes
Dantrolene	Yes
Felbamate	Yes
Flutamide	Yes
Gemtuzumab	Yes
Isoniazid	Yes
Ketoconazole	Yes
Naltrexone	N/A
Nevirapine	N/A
Pemoline	N/A
Tolcapone	N/A
Trovafloxacin	N/A
Valproic acid	Yes

Drug Name	Reactive Metabolite Formation
C. Drugs with a warning	
Acetaminophen	Yes
Carbamazepine	Yes
Clozapine	Yes
Diclofenac	Yes
Disulfiram	Yes
Halothane	Yes
Leflunomide	Yes
Methyldopa	Yes
Rifampin	Yes
Tacrine	Yes
Tamoxifen	Yes
Terbinafine	Yes
Ticlopidine	Yes
Zileuton	Yes

Drug Name	Reactive Metabolite Formation
D. Miscellaneous hepatotoxic drugs	
Alpidem	Yes
Amineptine	Yes
Amodiaquine	Yes
Cinchophen	N/A
Dihydralazine	Yes
Dilevalol	N/A
Ebrotidine	N/A
Glafenine	N/A
Ibufenac	Yes
Isaxonine	Yes
Niperotidine	N/A
Perhexiline	N/A
Pirprofen	Yes
Tilbroquinol	N/A

Common reactive drug metabolites associated with hypersensitivity drug reactions include: nitroso-SMX implicated in SMX hypersensitivity reactions (Cribb and Spielberg 1990), Glycinexylidide and monoethyl glycinexylidide are linked with lidocaine hypersensitivity reactions (Tam et al. 1990). Furthermore,

carbamazepine hypersensitivity is associated with its arene oxide and quinone metabolites (Pearce et al. 2002; Pearce et al. 2005) while N-chloro, N-oxide and arene oxides are implicated in lamotrigine reactions (Maggs et al. 2000; Lu and Uetrecht 2007).

In 2009, Stepan et al examined the physiochemical properties of the top 200 drugs in United States (Stepan et al. 2011). The authors reported that 78-86% of these drugs possessed structural alerts. Moreover, ADRs resulting from 62-69% of the drugs with structural alerts were due to reactive metabolites. Hence, a better understanding of the role of reactive metabolites in drug-induced idiosyncratic hypersensitivity is required.

1.7 Sulfamethoxazole antigenicity and immunogenicity

The molecular mechanisms involved in drug-induced immune responses are complex and not yet fully understood (Uetrecht 2007; Uetrecht and Naisbitt 2013). SMX is a drug used with trimethoprim as a combination therapy in a 5:1 ratio (co-trimoxazole) against *Pneumocystis carinii* in patients with HIV and in cystic fibrosis patients for the treatment of respiratory infections (Pirmohamed et al. 1998; Lavergne et al. 2010; Elsheikh et al. 2011). The mechanism of SMX action involves the competitive inhibition of dihydrofolate reductase which catalyses the formation of folic acid, a substrate essential for bacterial DNA synthesis (Hong et al. 1995; Kalkut 1998). High dose co-trimoxazole for prophylaxis or treatment of infections is associated with a high incidence of cutaneous reactions in patients with HIV and in those suffering from cystic fibrosis (van der Ven et al. 1991; Lavergne et al. 2010; Elsheikh et al. 2011). The incidence of ADRs following SMX administration in these patients is 30% compared with 1-3% in the general population.

Low levels of glutathione (30-60% less) and hyper-reactivity of the immune system have been implicated in the increased prevalence of hypersensitivity reactions to SMX in these patients groups (Buhl et al. 1989; Roederer et al. 1991). However, the disease itself may be the primary factor; increasing risk through the provision of enhanced co-stimulatory signalling.

SMX in the absence of metabolism is chemically inert and cannot interact covalently with macromolecules. Hepatic metabolism of SMX by cytochrome P450 enzyme generates a hydroxylamine metabolite. SMX hydroxylamine is not protein reactive and is excreted unchanged in human urine. Auto oxidation of SMX hydroxylamine however generates nitroso-SMX metabolites which modifies cysteine residues on proteins to form various antigenic epitopes for drug specific T-lymphocytes (Park et al. 1987; van der Ven et al. 1994; Naisbitt et al. 1996). Put together, a combination of factors related to the patient, SMX and the pathophysiology of the disease (HIV or cystic fibrosis) are critical to the clinical manifestations of SMX-hypersensitivity reactions. Therefore, experiments designed to study the molecular mechanisms and the immunological basis of drug hypersensitivity reactions should be all inclusive.

1.8 Aims and objectives of thesis

The aim of this thesis was to investigate various possible chemical, genetic and immunological factors that may either cause or influence SMX-hypersensitivity reactions. To delineate the antigenic determinants of SMX-hypersensitivity in patients with cystic fibrosis, we performed a battery of immunochemical, mass spectrometric, biological and functional experiments, using blood samples from HLA-typed human donors.

The specific aims of the thesis include:

- Investigation of the molecular mechanism of SMX.NO specific T-lymphocyte activation
- Evaluation of enzyme expression profiles in various immune cells
- Determination of HLA-restricted recognition of SMX.NO by drug-specific T-lymphocytes and the extent of alloreactivity
- Investigation of the antigenicity/immunogenicity of SMX.NO-modified MPO-derived peptides
- Utilization of an *in vitro* T-lymphocyte priming assay to define the drug antigen(s) that activate naïve T-cells
- To investigate the role of PD-1/PD-L1 signalling in drug-specific T-cell responses

Chapter 2: Characterization of enzyme expression and sulfamethoxazole metabolism in immune cells

Contents

2.1 Introduction

The causes of ADRs in humans are numerous and may be related to the chemical properties of the drug(s) involved, the genetic factors of the patient and the pathophysiology of the disease being treated. Phase I drug metabolism involves oxidation, reduction and hydrolysis. The outcome of Phase I metabolism is the addition of a functional groups which facilitates Phase II conjugation. Drug metabolism (Phase I and Phase II) facilitates the elimination of both parent drugs and their metabolites; however reactive metaolites are sometimes generated. The formation of reactive metabolites is often implicated in idiosyncratic ADRs (Shah et al. 1982; Park et al. 1992; Pirmohamed et al. 1994). The relationship between drug metabolism and idiosyncratic ADRs has been well studied (Schaffner 1975; Lennard 1993; Schnyder et al. 1997; Griem et al. 1998; Naisbitt et al. 2003; Onder et al. 2011).

Normally, a balance exists between drug bioactivation and detoxification systems, however, in certain pathologies like CF and HIV/AIDS, alterations of the redox status results in raised concentrations of reactive metabolites and an increased number of ADRs (Pirmohamed et al. 2000; Burkhart et al. 2001; Elsheikh et al. 2011). Figure 2.1 illustrates the role of drug metabolism in ADRs and a list of reactive metabolites thought to be involved in cell damage. The rate of drug clearance from the body is a vital determinant of an individual's drug plasma concentration as well as susceptibility to adverse reactions. This is important for drugs with narrow therapeutic windows like lithium, digoxin and phenytoin. The three major factors affecting drug metabolism have been identified as: (1) Genetic factors (e.g thiopurine methyltransferase, N-acetyltransferase and UDP-glucuronosyltransferase polymorphisms). (2)

Environmental factors (e.g cigarettes, alcohol and oral contraceptives). (3) Physiological factors (age, disease states, and gender).

Although the liver is the major organ involved in drug metabolism, the skin is the main target organ affected in ADRs. The stability of reactive metabolites in general circulation from the organs where they are generated (liver) to distal organs like the skin has been questioned (Reilly et al. 2000; Irving and Elfarra 2012). Therefore, localised generation of reactive metabolites at specific sites like the skin could be the trigger for the pronounced clinical involvement of the skin in such ADRs.

Cutaneous adverse reactions are the major clinical manifestations of sulfamethoxazole hypersensitivity reactions (Carr et al. 1993; Carr et al. 1994) and are thought to be immune mediated involving drug-specific T-lymphocytes (Schnyder et al. 1997; Schnyder et al. 2000; Burkhart et al. 2001; Burkhart et al. 2002).

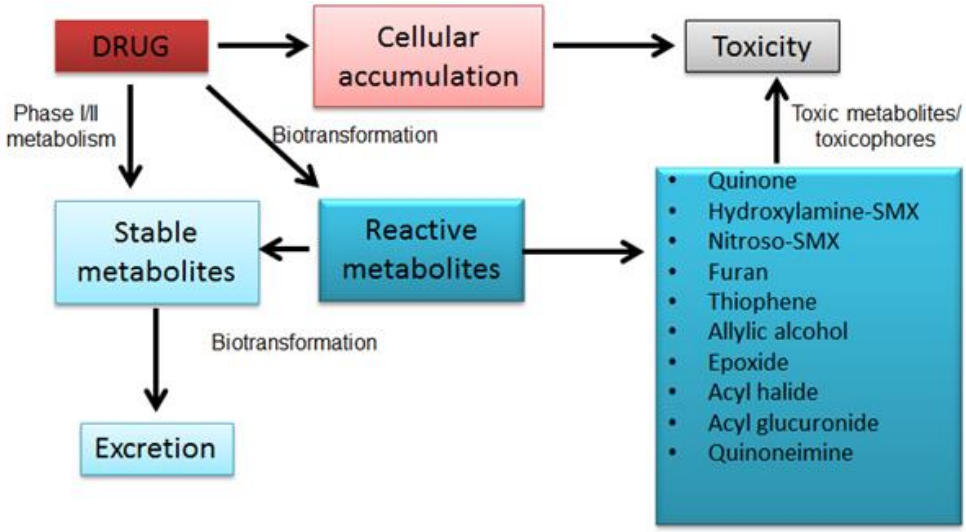


Figure 2.1-Schematic representation of drug metabolism pathways and some examples of chemically reactive metabolites involved in ADRs.

SMX largely undergoes N-acetylation in the liver to generate non-toxic metabolites which are safely eliminated (Cribb et al. 1993; Uetrecht and Naisbitt 2013). A small amount of SMX is converted by CYP2C9 and myeloperoxidase to SMX hydroxylamine (SMX.NHOH) (van der Ven et al. 1994; Cribb et al. 1995). SMX.NHOH is stable, enters the systemic circulation and can be excreted unchanged in the urine (Cribb and Spielberg 1992; van der Ven et al. 1994; Gill et al. 1996). SMX.NHOH is not protein-reactive but readily undergoes auto-oxidation to form the highly protein-reactive nitroso-sulphamethoxazole, SMX.NO (Cribb et al. 1991; Naisbitt et al. 1996; Naisbitt et al. 2001; Naisbitt et al. 2002; Summan and Cribb 2002). SMX.NO reacts with SMX.NHOH to form azo and azoxy dimers (Naisbitt et al. 1996). Both dimers are incapable of activating T-lymphocytes. Further oxidation of SMX.NO generates nitro SMX (Naisbitt et al. 2002). SMX.NO-modified proteins (intracellular or extracellular) are then, degraded into peptide fragments and presented in an MHC class II restricted manner to $CD4^+$ T-lymphocytes (Manchanda et al. 2002; Elsheikh et al. 2011).

The possibility of SMX.NHOH being transported as a non-reactive intermediate and only oxidised under condition of stress such as HIV/AIDS and cystic fibrosis has been reviewed elsewhere (Uetrecht and Naisbitt 2013). SMX.NO stimulates both the innate and the adaptive immune systems through the activation of dendritic cells and T-lymphocytes, respectively via protein haptenation (Sanderson et al. 2007).

The DCs that are resident in the skin (Langerhans' cells) are often in a quiescent state and continuously sample the environment for foreign organisms. The major phenotypic difference between Langerhans's cells and blood derived DCs is the expression of cutaneous leucocyte antigen (CLA) in the former (Yasaka et al. 1996). CLA expression directs the homing precursor DCs in the blood destined to become Langerhans cells to the skin. Migratory Langerhans' cells have up-regulated MHC II, CD40 and DC86 in a similar manner as mature blood-derived dendritic cells (Rajkovic et al. 2011).

Independent groups have suggested that reactive metabolites are generated locally at specific target tissues such as the skin by keratinocytes (Reilly et al. 2000; Vyas et al. 2006; Vyas et al. 2006). SMX.NO reduction by ascorbate and glutathione protects cell systems from high levels of reactive metabolites and subsequent hypersensitivity reactions (Cribb et al. 1995; Kurian et al. 2004). The hepatic metabolism profile of SMX is illustrated in figure 2.2 below.

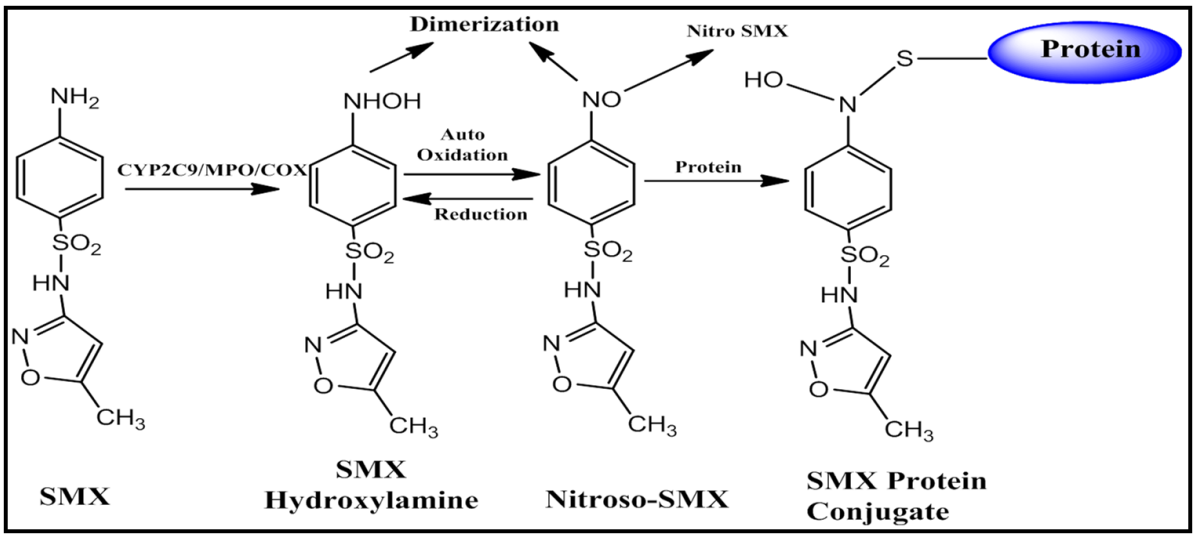


Figure 2.2-Schematic representation of SMX metabolism

2.2 Aims

The molecular mechanism of T-lymphocyte activation by SMX and SMX.NO has been researched extensively. While the hepatic metabolism of SMX has been well characterised, it is still not clear whether the reactive metabolite (SMX.NO) generated within the liver circulates to the skin where it causes cutaneous reactions, or whether localised cutaneous generation of protein reactive metabolites are responsible for these reactions in the skin. We hypothesised that SMX metabolism by immune cells may be crucial for the drug-induced cutaneous reactions Therefore, the aims of this chapter were:

- To determine enzyme expression in immune cells
- To investigate SMX metabolism in immune cells
- To analyse the role of SMX immune cell metabolism in the activation of T-cells

2.3 Methods

2.3.1 Chemicals and reagents

Foetal bovine serum (FBS) and MPO activity assay kit was purchased from Invitrogen (Paisley, UK). SMX, anti-rabbit IgG peroxidase secondary antibody, monoclonal anti-β-Actin antibody, DMSO, Tween-20, skimmed dry milk, alkaline phosphatase-conjugated anti-rabbit antibody produced in mouse, alkaline phosphatase substrate, phytohemagglutinin (PHA), HL60 cells (neutrophil-derived cell line), Coomassie Blue G-250, methimazole, ascorbic acid, ammonium formate, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Bradford reagent was obtained from BIO-RAD (Hempstead, UK). Nitroso-SMX ($C_{10}H_9N_3O_4S$) was purchased from Dalton chemical laboratories Inc. (Toronto, Canada). Anti-SMX antibody produced in rabbit was developed by Panigen (Blanchard Ville, USA). Tritiated [^{3}H]-methyl thymidine was purchased from Moravek (California, USA). High capacity protein binding micro titre plates were purchased from Falcon, BD Bioscience (Oxford, UK). Microplate reader (MRX) was manufactured by Dynatech Laboratories Inc., Chantilly, VA, USA). RNeasy mini kit and RNase-free DNase kit were bought from Qiagen (Crawley, UK). TaqMan primers, PCR reagents, reverse transcription reagents were purchased from Applied Biosystems, Warrington, UK. Reducing Laemmli sample buffer was purchased from Bio-Rad (Hertfordshire, United Kingdom). Chemiluminescent substrate was bought from Thermo Scientific (Northumberland, United Kingdom). Nitrocellulose membrane was purchased from GE Healthcare Life Sciences (Buckinghamshire, United Kingdom). RNeasy Mini kit and RT2-First Strand kit were bought from (QIAGEN Ltd. Crawley, U.K.). HPLC grade methanol, analytical

grade acetonitrile and HPLC grade distilled water were obtained from Fisher Scientific (Loughborough, UK). Interferon-γ ELISpot kits including antibodies and substrate solution were purchased from Mabtech (Stockholm, Sweden). ELISpot plates were bought from Millipore Corporation (Millipore, Watford, UK).

2.3.2 Cell culture medium

Culture medium for T-lymphocytes comprised of RPMI 1640 supplemented with 10% human AB serum, HEPES (25 mM), penicillin (1000 U/ml), streptomycin (0.1 mg/ml), L-glutamine (2 mM) and transferrin (25 μg/ml).

EBV-transformed B-cells were maintained in medium comprised of RPMI 1640 supplemented with 10% foetal bovine serum, HEPES (25 mM), penicillin (1000 U/ml), streptomycin (0.1 mg/ml) and L-glutamine (2 mM).

Dendritic cells were cultured in medium containing RPMI-1640, penicillin (100 μg/ml), streptomycin (100 U/ml), transferrin (25 μg/ml), 10% human AB serum, HEPES buffer (25 mM), and L-glutamine (2 mM); supplemented with GM-CSF (800 U/mL) and IL-4 (800 U/mL).

HL60 cell line was maintained in HL60 medium composed of RPMI-1640 supplemented with 10% foetal bovine serum and L-glutamine (2 mM).

2.3.3 Isolation of peripheral blood mononuclear cells (PBMCs) and generation of drug specific T-cell clones

Blood (50 mL) was collected from 3 subjects for generation of EBV-transformed B-cells. Approval for the study was obtained from the Liverpool local research ethics committee and informed written consent was obtained from patients. PBMCs were isolated from blood collected in heparinised vacutainer tubes.

Blood (25 mL) was carefully layered on top of lymphoprep (25 ml) and spun in a centrifuge (2000 r.p.m, 25 mins, and 25°C). The buffy coat layer containing the PBMCs was carefully collected using a Pasteur pipette. PBMCs were washed twice in Hanks balanced salt solution (HBSS) to remove any remaining lymphoprep and the pellet was resuspended in 10 ml HBSS.

An aliquot of PBMC suspension (10 µL) was added to an equal volume of trypan blue (0.2 % w/v) and the cells were counted using a Neubauer haemacytometer (Sigma-Aldrich) under a Leica DME microscope (Leica Microsystems, Milton Keynes). Cell viability was evaluated by trypan blue exclusion of viable cells. The percentage viability was estimated as follows: **percentage viability = viable cells ÷ total cells × 100.** Percentage viability was ≥95% for all the PBMC isolations carried out. PBMCs were again spun down and resuspended in foetal bovine serum containing 10% DMSO at a density of 10^7 cells/ml for 24 hours in a Mr Frosty at -80 ˚C and then frozen at -150˚C for long term storage. Figure 2.3 illustrates the steps involved in PBMC isolation.

PBMCs (1×10^6/well; 0.5 ml) from hypersensitive patients were cultured with either SMX (1 mM and 2 mM) or SMX.NO (25 µM and 50 µM). On days 5 and 9, culture medium was supplemented with IL-2 (200IU/ml) to expand the number of antigen specific T-cells prior to cloning on day 14. Autologous Epstein-Barr virus (EBV)-transformed B-cell lines were used as antigen presenting cells in assays with clones.

Antigen-specificity was assessed by culturing irradiated EBV-transformed B-cells (1×10^4 cells/well) and either SMX (2 mM) or SMX.NO (50 µM) with T-cell clones (5×10^4 cells/well; 200 µl) for 48 hours. Proliferation was measured by

the addition of [^{3}H] thymidine followed by scintillation counting. Clones with a stimulation index of greater than 2 were expanded by repetitive stimulation with irradiated allogeneic PBMCs (5×10^5 cells/well) and PHA (5 µg/ml) in IL-2 containing medium. Five SMX.NO responsive TCCs generated from 1 SMX hypersensitive patient were used to define the role metabolism in SMX hypersensitivity. The phenotype and specificities of the other drug specific TCCs generated from 4 other hypersensitive patients are discussed in chapters 3 and 6.

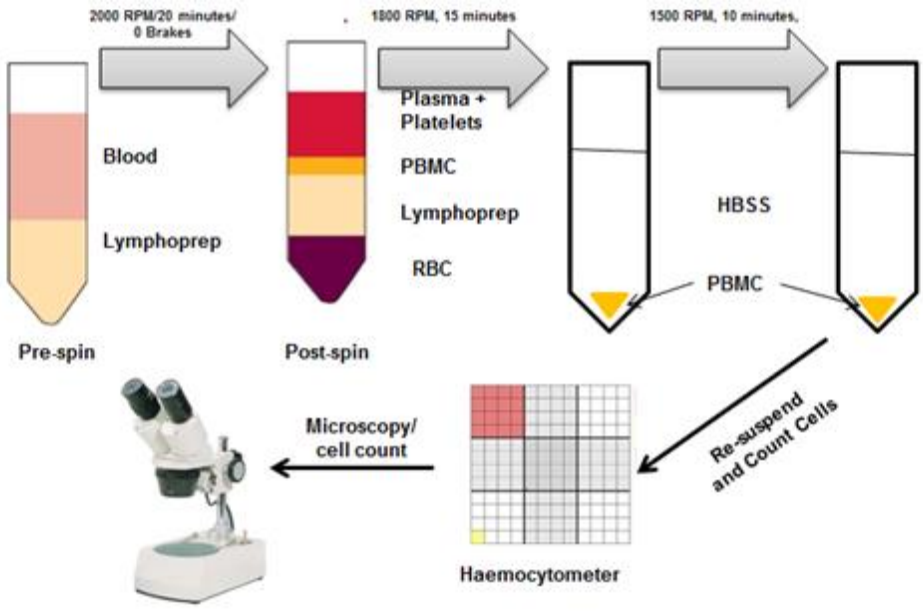


Figure 2.3–Schematic representation of PBMC isolation from whole blood.

2.3.4 Generation of dendritic cells (DCs)

CD14$^+$ monocytes were isolated from PBMCs using magnetic beads and columns according to the manufacturer's instructions (Miltenyi Biotech; Bisley, UK) and then cultured in dendritic cell culture medium for 7-8 days to encourage

differentiation to DCs. The expression of CD14, CD40, CD86 and MHC II were determined using flow cytometry.

2.3.5 Generation of Epstein-Barr virus transformed B-cells

PBMCs were transformed into B-cell lines using supernatant from the virus-producing cell line B9.58. PBMCs (5×10^6) were resuspended in supernatant from B9.58 cell (5 ml) pre-filtered with a 0.45 µm syringe filter. Cyclosporin A (CSA, 1 ug/ml) was added in order to inhibit the proliferation of T-lymphocytes and PBMCs were incubated overnight at a temperature of 37°C under an atmosphere of 95% O_2/5% CO_2. Cells were then washed and resuspended in APC medium supplemented with CSA (1 µg/ml) and cultured in a 24-well plate. Fresh APC culture medium was added twice a week to maintain the cells. CSA was omitted from the culture medium after two weeks to enhance the proliferation of the B-cells. Cells were transferred to a tissue culture flask when confluent and maintained with fresh APC culture medium twice a week.

2.3.6 Antigen presenting cell fixation and antigen pulsing assays

The role of intracellular SMX metabolism by immune cells in the activation of T-lymphocytes was investigated using APC fixation. Autologous EBV-transformed B-cells (2×10^6 cells/ml) were washed twice in HBSS to exclude FBS and resuspended in HBSS (1 ml). Glutaraldehyde (25%, 1 µL) was then added and the cells were gently mixed for 30 seconds. Glycine (1ml of 1 M) was quickly added and cells were mixed for a further 45 seconds. Cells were washed three times to remove glutaraldehyde and were then resuspended in T-lymphocyte culture medium. T-cell clones (5×10^4) were co-cultured with glutaraldehyde-fixed EBV-transformed B-cells (1×10^4 cells, 50 µL) in the presence or absence of

SMX. In other experiments, antigen-presenting cells (APCs) pulsed with SMX for 16 hours and washed extensively with HBSS to exclude free SMX were co-incubated with SMX.NO-specific T-cell clones in a 96-well U-bottom microplate for 48 hours under an atmosphere of 95% O_2/5% CO_2 at 37˚C. [^{3}H]-thymidine was added for the final 16 hours of incubation and T-lymphocyte proliferation was evaluated using scintillation counting. Soluble SMX.NO was used as a positive control.

2.3.7 Cell lysis and protein quantification

EBV-transformed B-cells, dendritic cells or HL60 cell lines (2×10^6 cells/ml) were cultured with either SMX (0.5 -2 mM) or SMX.NO (5-50 uM) in a 24 well culture plate at 37°C under an atmosphere of 95% O_2/5% CO_2 for 16 hours. The cells were washed three times with HBSS (by centrifugation at 1500 rpm for 5 minutes) to remove non-covalently bound drugs. Cell pellets were re-suspended in 200 µL RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 2.5mM EDTA, 10% (w/v) Glycerol, 1% (w/v) Triton X-100, 1mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1mM PMSF, 0.1% (w/v) SDS, and 0.5% (w/v) Na deoxycholate) and placed on ice for 30 minutes to lyse. Cells were given three bursts of sonication while maintaining them on ice. The cell suspensions were then spun down at 14,000 × g for 10 minutes at 4°C. Supernatants were then collected and protein concentration determined using the well-established Bradford assay (Bradford 1976). Protein lysates were then standardised to 250 µg/ml. A standard calibration curve was prepared using BSA (0-2000 µg/ml). Briefly, 10 µL of either standard or test samples were plated unto a 96-well flat-bottom microplate in duplicates. Bradford reagent (200 µL) was added to all the wells. The plate was protected from light and the absorbance was read at 570

nm using a microplate reader (Dynex Technologies, Billinghurst, West Sussex). The protein concentration of each sample was determined using the standard curve generated from BSA.

2.3.8 Enzyme-linked immunosorbent assay (ELISA) to measure SMX-derived adducts formed in immune cells

Cell lysates (100 µL, 250 µg/ml) were plated in duplicate onto a high capacity protein-binding microtitre 96-well ELISA plates and incubated for 16 hours at 4°C. Wells were washed (5 times) with phosphate-buffered saline (PBS recipe) containing 0.001% Tween-20. Wells were incubated in 2.5% skimmed milk prepared using PBS-Tween for 1 hour to block non-specific antibody binding sites. Wells were then washed 5 times with PBS-Tween and incubated in 100 µL anti-SMX antibody (1:2000) overnight at 4°C. Anti-SMX antibody was generated according to previously reported methods (Gruchalla and Sullivan 1991; Lavergne et al. 2006). After overnight incubation, wells were washed 5 times and then incubated in 100 µL alkaline phosphatase-conjugated anti-rabbit IgG (1:1000) for 2 hours. This was followed by 5 washes and 30 minutes incubation in alkaline phosphatase substrate (100 µL/well). Spectrophotometric absorbance was determined using a Microplate reader (MRX) at an optical density (OD) of 405 nm. Readouts from ELISA were calculated using the formula: **Absorbance of Sample (Δ OD) = Sample OD – Vehicle OD.** Figure 2.4 is a schematic representation of an indirect ELISA to detect SMX/SMX.NO-modified protein.

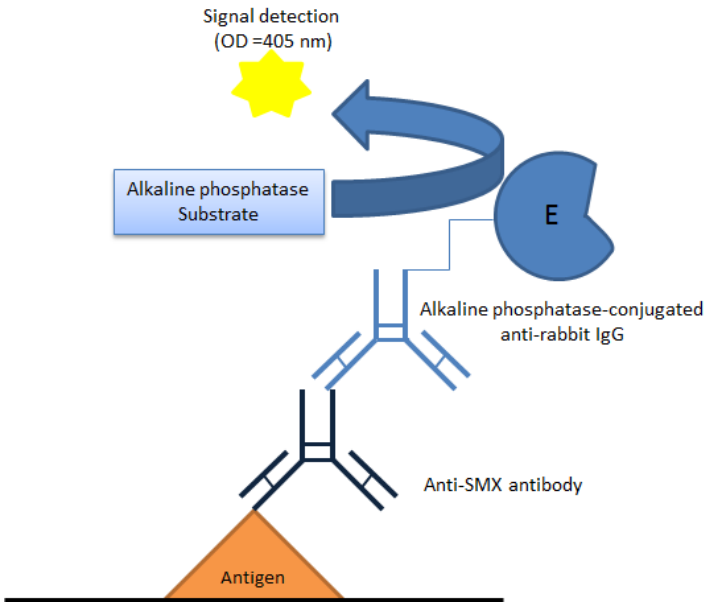


Figure 2.4-Schematic representation of an indirect ELISA to detect SMX/SMX.NO-modified protein using an anti-SMX specific primary antibody and an enzyme conjugated secondary antibody.

2.3.9 Coomassie Blue staining

Protein lysates (10 µl/lane) were separated using a 12% SDS-PAGE gel (300 V, 60 mA, 1 hour) and the gel was fixed in 7% glacial acetic acid in methanol (40% v/v). It was then placed in Coomassie Blue G-250 (0.1%w/v) prepared in 20% methanol and agitated in staining suspension. After 1 hour, the gel was de-stained using 10% acetic acid in methanol (25% v/v) for 1 minute with gentle agitation. The gel was then rinsed with 25% methanol and de-stained in fresh 25% methanol for up to 24 hours. Bands of interest were excised followed by in-gel tryptic digestion and proteomic analysis. Briefly, bands excised from Coomassie Blue-stained gels were de-stained by adding 100 µl 50% ACN/50mM ammonium bicarbonate and incubating for 15mins at room temperature with occasional agitation. The supernatants were discarded and the gel bands were dried in a SpeedVac (15-20mins). They were rehydrated in 100 µL 10 mM

dithiothreitol/50 mM ammonium bicarbonate and incubated at 56°C for 1 hour. The supernatants were removed and the bands were incubated in 55 mM iodoacetamide/50 mM ammonium bicarbonate (25 µL) for a further 45 minutes in the dark. The gel pieces were washed with ammonium bicarbonate for 10 minutes before being dried once more in a SpeedVac. They were then rehydrated in 100 µL 10 ng/µL trypsin/50 mM ammonium bicarbonate and incubated overnight at 37°C. In order to extract the peptides from the gel pieces, they were incubated in a sonicator bath in 30 µL 60% acetonitrile/1% trifluoroacetic acid for 5 mins. After brief centrifugation, the supernatants were collected. This step was repeated once more, and then the supernatants were pooled and dried in a SpeedVac. The peptides were resuspended in 10 µL 0.1% formic acid, and 0.5 µL was spotted onto a MALDI target plate or 5 µL was analysed by LC-MS.

2.3.10 Western blotting

Protein lysates (10 µL/lane) were prepared in RIPA buffer. Lysates were treated with reducing Laemmli buffer and then separated by electrophoresis on a 12% SDS-PAGE gel (300 V, 60 mA, 1 hour). Separated proteins were then transferred from the gel onto a nitrocellulose membrane (300 V, 250 mA, and 1 hour). Membranes were then blocked in 2.5 % (w/v) skimmed milk prepared in TST buffer (150 mM NaCl, 50mM Tris-HCl, pH 7.6 0.05% Tween-20). Immunodetection of SMX–protein adducts was performed by incubating the blot with anti-SMX rabbit antiserum (1:2000) in phosphate buffer overnight at 4 °C. Unbound antibody was removed by washing with PBS-Tween, and the membrane was incubated with peroxidase-conjugated anti-rabbit IgG antibody (1:10,000 in TST buffer) for 2 hours at room temperature. The membrane was

finally developed using an enhanced chemiluminescent substrate. Figure 2.5 illustrates a schematic representation of the Western blotting protocol utilised.

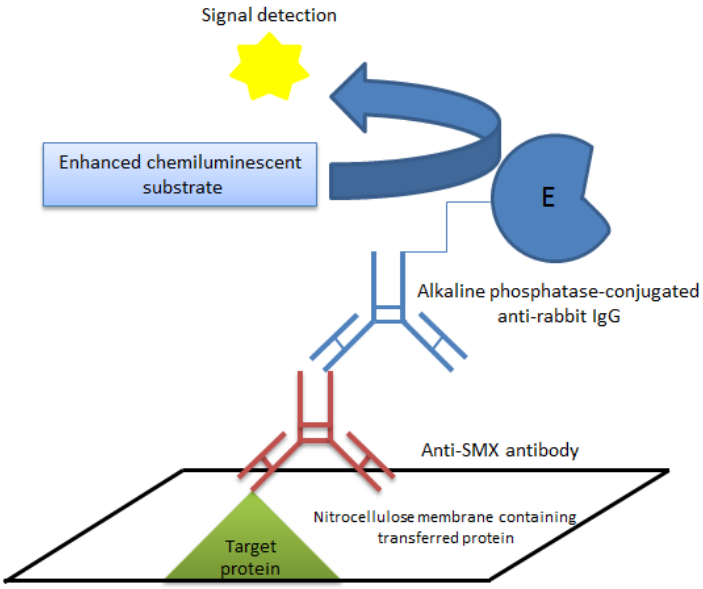


Figure 2.5-Schematic representation of the Western blotting used to detect SMX/SMX.NO-modified protein adducts using an anti-SMX specific primary antibody, an enzyme conjugated secondary antibody and enhanced chemiluminescent substrate.

2.3.11 Real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from EBV-transformed B-cells (5×10^6), HL60 cell line (5×10^6) and dendritic cells (5×10^6) with the RNeasy Mini kit. Using a Nano Drop spectrophotometer (Thermo Scientific, Surrey, U.K.), the concentrations of RNA in the samples were measured. RNAs from various samples were then reverse transcribed to cDNA using the RT^2-First Strand kit. RNA (1 µL) was incubated with 2 µL of genomic DNA elimination reagents at 42°C for 5 mins. A reverse transcription cocktail (10 µL) was added to equal volume of genomic DNA elimination mixture and incubated at 42°C for 15 minutes. The reaction was brought to an end by raising the temperature to 95°C for 5 minutes. RNase-free

water (91 µL) was added to the resulting cDNA (20 µL) and then centrifuged to ensure a homogenous mixture. Experimental cocktail (1350 µL of Mastermix, 102 µL of cDNA, and 1248 µL of water) was prepared and 25 µL/ well was added to a PCR array plate and tightly sealed with a transparent microplate sealer. A two-step thermal cycling was performed using an ABI 7000 thermal cycler (Applied Biosystems, Foster City, USA); 1 cycle at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. SYBR green was detected and recorded for each well. Relative quantification was achieved by determining the cycle at which the fluorescence reached a threshold value (C_t).

2.3.12 Proteomic analysis of drug metabolism enzyme in immune cells

Proteins were extracted from a pooled sample of EBV-transformed B-cells cultured and expanded in T-175 flasks (Thermo Scientific (Northumberland, United Kingdom) by three cycles of sonication in triethylammonium bicarbonate (0.5 M)/ SDS (0.1%). One cycle of freeze–thawing at −80 °C and a further round of sonication completed the cell lysis. The cell suspension was centrifuged at 14 000 × g and 4 °C for 10 minutes. Protein supernatants were collected and the protein concentration was determined by Bradford assay (Bradford 1976). Protein was reduced by incubation with dithiothreitol (10 mM) for 15 mins at room temperature followed by alkylation with iodoacetamide (55 mM) for 15 mins. Proteins were digested overnight with trypsin at 37 °C, and the tryptic peptides were fractionated by strong cation exchange chromatography using a previously described method (Jenkins et al. 2009). A gradient from 0 to 0.5 M KCl in KH_2PO_4 (10 mM)/ ACN (25%), pH 3,

was applied at a flow rate of 1 ml/minute for 20 minutes and 2 mL fractions were collected. The fractions were evaporated to dryness in a SpeedVac (Eppendorf Ltd., Cambridge, U.K.) reconstituted in trifluoroacetic acid 0.1% (v/v) and desalted using a macroporous C_{18} High-Recovery reversed phase column measuring 4.6 mm × 50 mm (Agilent Technologies) installed on a Vision workstation (AB Sciex). Desalted samples were dried once more and were reconstituted in 20 µL of 0.1% formic acid just prior to LC-MS/MS analysis.

Samples were injected into a Triple TOF 5600 mass spectrometer (AB Sciex) by automated in-line reversed phase liquid chromatography, using an Eksigent NanoUltra cHiPLC System mounted with microfluidic trap and analytical column (15 cm ×75 µm) packed with ChromXP C_{18}-CL 3 µm. A NanoSpray III source was fitted with a 10 µm inner diameter PicoTip emitter (New Objective, Woburn, MA). Samples loaded onto the trap were washed with acetonitrile (2%)/formic acid (0.1%) for 10 minutes at 2 µL/min before switching in-line with the analytical column. A gradient of 2–50% ACN/0.1% formic acid over 90 mins was applied to the column at a flow rate of 300 nL/min. Spectra were acquired automatically in positive-ion mode using information-dependent acquisition powered by Analyst TF 1.5.1 software, across mass ranges of 400–1600 amu in MS and 100–1400 amu in MS/MS. Up to 25 MS/MS spectra were acquired per cycle (approx. 10 Hz) using a threshold of 100 counts per second, with dynamic exclusion for 12 s and rolling collision energy. Database searching was performed using Protein Pilot software version 4 (AB Sciex), with the confidence set to 10% to enable searching of the reversed decoy database. Carboxamidomethyl modification of cysteine residues and biological modifications were allowed. Data were searched against the latest version of the

SwissProt database, and only proteins falling within a 1% global false discovery rate were included in the results.

2.3.13 HPLC determination of SMX metabolites in immune cells

EBV-transformed B-cells, HL60s and DCs were incubated with SMX (2 mM) for 16 hours at 37°C under an atmosphere of 95% O_2/5% CO_2. Reactions were terminated after 16 hours with the addition of an equal volume of ACN and protein was precipitated at -20°C overnight. After centrifugation (2200 rpm, 10 mins) of samples, supernatants were loaded onto Sep-Pak C-18 solid phase extraction cartridges (Waters Ltd, Herts, U.K.). Cartridges were then washed with 3 ml of distilled water and metabolites were eluted with 3 ml MeOH. The MeOH fractions were evaporated to dryness under a steady stream of N_2 at room temperature. Samples were then reconstituted in MeOH: dH20 (50:50, 250µl: 250µl). Aliquots (50µl) of the reconstituted samples were injected onto HPLC.

Sample suspensions were resolved on a Gemini NX 5-µm C-18 column (250 x 4.60mm; Phenomenex, Macclesfield, Cheshire, U.K.) with ACN as the eluent (10% for 5 mins, increasing to 15% between 5 and 10 mins, and up to 50% between 20 and 40 mins) in ammonium formate (10 mM; pH 4.8). Eluents were delivered with a Dionex Summit HPLC System at a flow rate of 1ml/min through a UVD170S UV detector set at 254 nm (Dionex). Data was processed by Chromeleon software (Dionex). Using Chromeleon software, the percentage turnover and percentage metabolite formation was calculated from the area under the curve (AUC) from each trace. Authentic standards for SMX, SMX.NHOH, and SMX were used to identify metabolites.

2.3.14 ELISpot Assay

ELISpot plates were coated with 100 µL/well of interferon gamma capture antibody (15 µg/ml) and incubated overnight at 4°C. Wells were washed five times with sterile PBS and then blocked with 200 µL of T-lymphocyte culture medium for 30 minutes at room temperature. Drug specific T-cell clones (5×10^4, 50 µL) were added to wells alongside autologous irradiated EBV-transformed B-cells (1×10^4, 50µL). Cells were cultured in the presence or absence of SMX.NO (50 µM, 100 µL) and plates incubated at 37˚C under an atmosphere of 95% O_2/5% CO_2 48 hours. Cells were discarded after 48 hours and wells washed five times with 200 µL PBS. Biotin-labelled detection antibody was diluted to 1 µg/ml in PBS containing 0.5% FBS and 100 µL added to the wells. The plate was incubated at room temperature for 2 hours at room temperature. After 2 hours, wells were washed five times with PBS. Streptavidin-ALP diluted PBS containing 0.5% FBS (1:1000) was added to wells and incubated for 1 hour at room temperature. Wells were then washed five times with PBS (200 µL), and BCIP/NBT substrate (100 µL/well) was added for 15 minutes at room temperature in the dark. Wells were inspected for the development of spots and then washed under slow running tap water. A schematic of the ELISpot assay is represented on figure 2.6. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader (Cadama Medical, Stourbridge, UK).

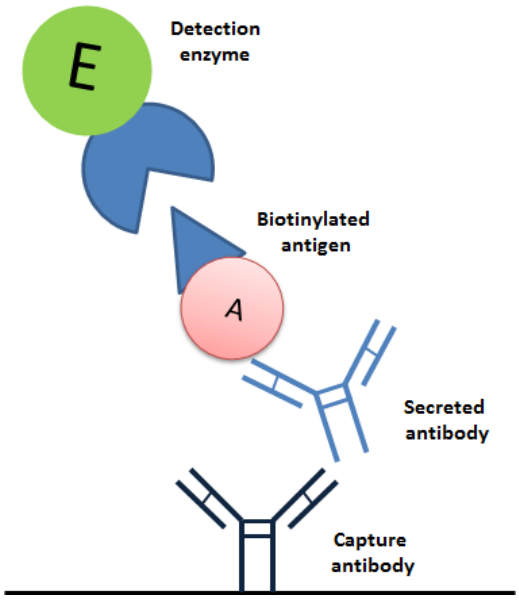


Figure 2.6– Schematic representation of the ELISpot assay to detect cytokine secretion.

2.3.15 Myeloperoxidase activity assay

A peroxidation activity assay showing MPO activity in HL60, DCs and EBV-transformed B cell lines was performed. Clarified cell lysate samples (50 µL) and 50 µL standard MPO (0-200 ng/mL) were plated into a 96-well microplate in duplicate. An equal volume of 2X Amplex Ultra Red reagent™ working solution was added to all samples and standards. The microplate was then incubated at room temperature for 30 minutes and protected from light. The reaction was stopped with 10 µL/well of peroxidase inhibitor (10X). The fluorescence intensity of each sample was then measured using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Background fluorescence intensity of zero MPO was subtracted from all the experimental samples and standards. The MPO concentration was then determined using the standard curve.

2.3.16 Statistical analysis

Mean values and standard deviations were calculated, and statistical analysis was performed using paired T tests (Sigma plot 12 software).

2.4 Results

2.4.1 Evidence of SMX metabolism and enzyme expression in immune cells

DCs generated from CD14+ monocytes expressed low levels of CD14 but high levels of costimulatory molecules like CD40, CD86 and MHC II (figure 2.7A). Metabolism of SMX by various immune cells was investigated using an immunochemical assay (ELISA). Differential metabolism of SMX was observed in various immune cells. The HL60 cells showed an approximately 3 fold increase in SMX.NO-modified protein when compared with either EBV-transformed B-cells or dendritic cells (figure 2.7B). Furthermore, the levels of drug-protein adduct formation increased with an increase in the concentrations of either SMX.NO or SMX during the incubation (figure 2.7C and D).

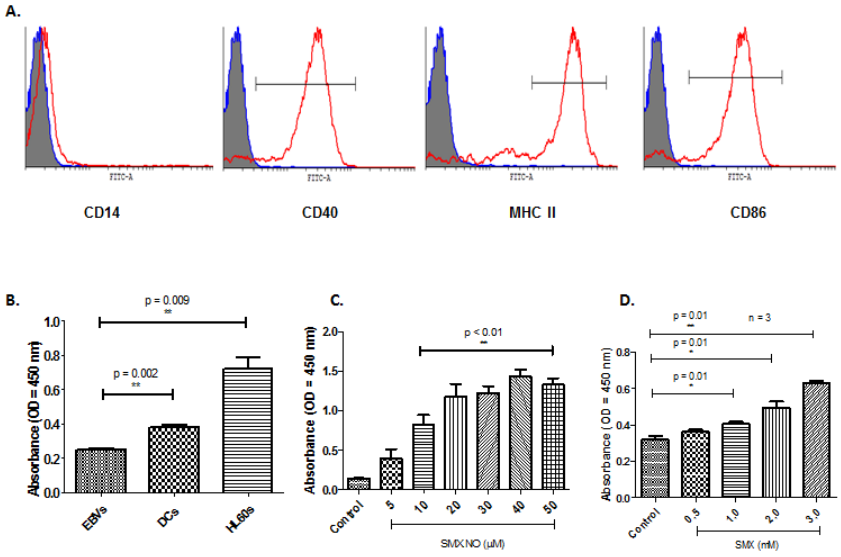


Figure 2.7 Sulfamethoxazole metabolism in immune cells. (A) Expression of co-stimulatory molecules on DCs. Grey histograms with blue boundaries represent unstained cells while red lines represent the co-stimulatory molecule expressed. **(B)** EBV-transformed B-cells, dendritic cells or HL60 cells (2×10^6/mL) were incubated with SMX (2 mM) for 16 hours at 37°C under an atmosphere of 95% O_2/5% CO_2. Cells were then harvested and lysed in RIPA buffer. ELISA was performed on protein lysates to determine levels of SMX.NO-modified protein. **(C and D)** EBV-transformed B-cells (2×10^6/mL) were incubated in either SMX.NO (5-50 μM) or SMX (0.5-3 mM) for 16 hours. Cells were then harvested, lysed and ELISA performed to determine levels of SMX.NO-modified adducts.

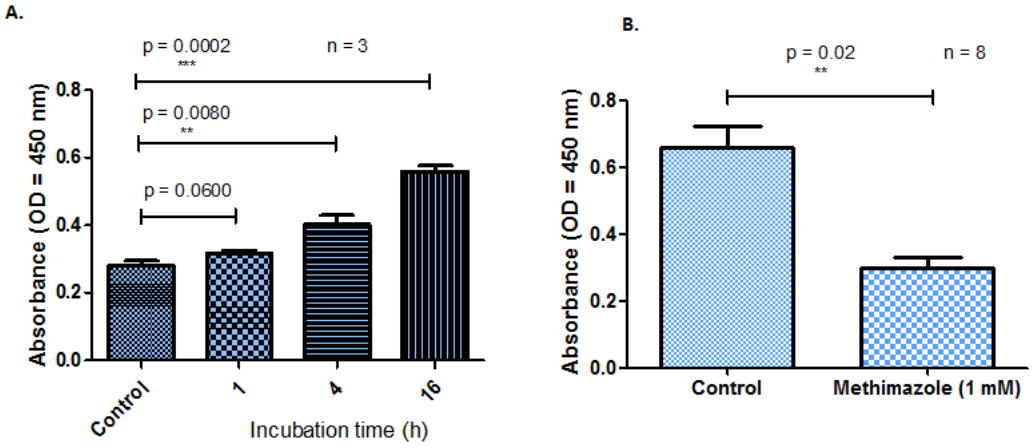


Figure 2.8 (A) Time-dependent SMX-protein adduction. EBV-transformed B-cells (2×10^6/mL) from three subjects were incubated in SMX (2 mM) for 1 hour, 4 hours and 16 hours under an atmosphere of 95% O_2/5% CO_2. Cells were harvested at the specified time points, lysed and protein concentration determined using the Bradford assay. ELISA was performed to determine the levels of SMX.NO-protein adduction. **(B)**. **Methimazole inhibition**. EBV-transformed B-cells (2×10^6/mL) from 8 individuals were pre-incubated with methimazole (1 mM) for 30 minutes followed by a 16 hour incubation with SMX (2mM). Cells were harvested, lysed and ELISA performed to determine the extent of SMX.NO-protein haptenation.

SMX metabolism by EBV-transformed B-cells was time-dependent with an optimum metabolism time found to be 16 hours (P = 0.0002) as shown in figure 2.8A. However, there was a significant difference in SMX-Protein adduct formation when EBV-transformed B-cell lines were pre-incubated in methimazole for 30 minutes before incubation with SMX for 16 hours (figure 2.8B)

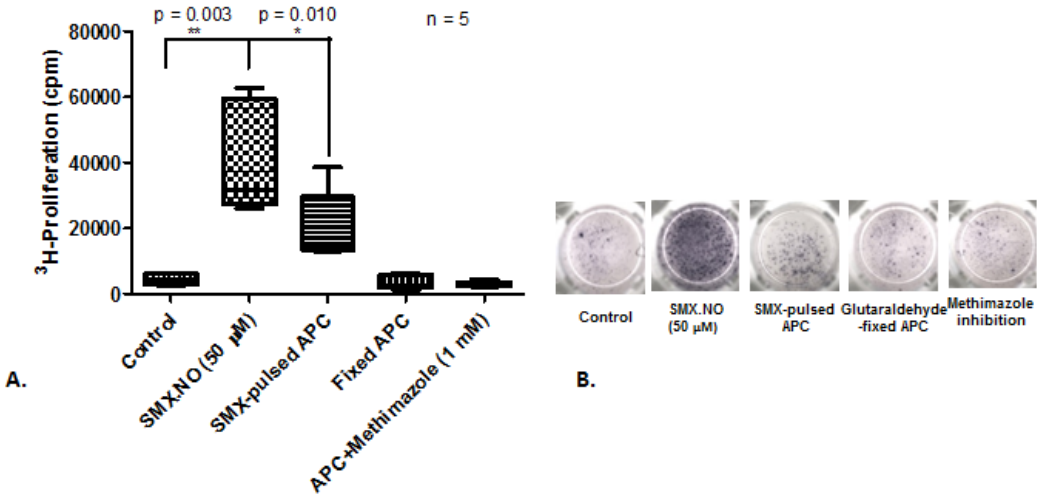


Figure 2.9 (A) SMX metabolism and enzyme inhibition in EBV-transformed B-cells. SMX.NO-specific T-cell clones (5×10^4 cells, 50 µL) were co-incubated with EBV-transformed B-cells (1×10^4 cells, 50 µL) in the presence and absence of soluble SMX.NO (50 µM, 100 µL) for 48 hours under an atmosphere of 95% O_2/5% CO_2. In other conditions, EBV-transformed B-cells were pulsed with SMX (2mM) for 16 hours, washed three times to remove unbound SMX and co-cultured with SMX.NO-specific T-cells for 48 hours. Furthermore, EBV-transformed B-cells that were either fixed with glutaraldehyde (25%) or pre-treated with methimazole (1 mM) for 30 minutes were incubated with SMX (2 mM) for 16 hours. EBVs were washed to remove unbound SMX and then co-cultured with SMX.NO-specific T-cells for 48 hours. T-cell proliferation was determined by [^{3}H]-thymidine incorporation. Data represent mean of duplicate wells. **(B)** Interferon-gamma ELISpot was performed with the same cell number and drug concentrations as stated in the proliferation assay (n= 3). Cells were incubated for 48 hours and spots developed according to the manufacturer's instructions.

EBV-transformed B-cells pulsed with SMX (2 mM) for 16 hours induced lymphocyte proliferation and interferon-gamma secretion consistent with antigen processing. APC either fixed with glutaraldehyde (25%) or pre-treated with methimazole (1 mM) before 16 hour incubation with SMX (2 mM) failed to induce either T-lymphocyte proliferation or interferon-gamma secretion (figure 2.9).

2.4.2 HPLC determination of SMX metabolism by antigen presenting cells

To further investigate SMX metabolism in APCs, a HPLC method was set up to track SMX metabolites in EBV-transformed B-cells. Cell supernatant and whole

cell lysates were processed and analysed using HPLC. Metabolites consistent with SMX metabolism could not be determined using HPLC method.

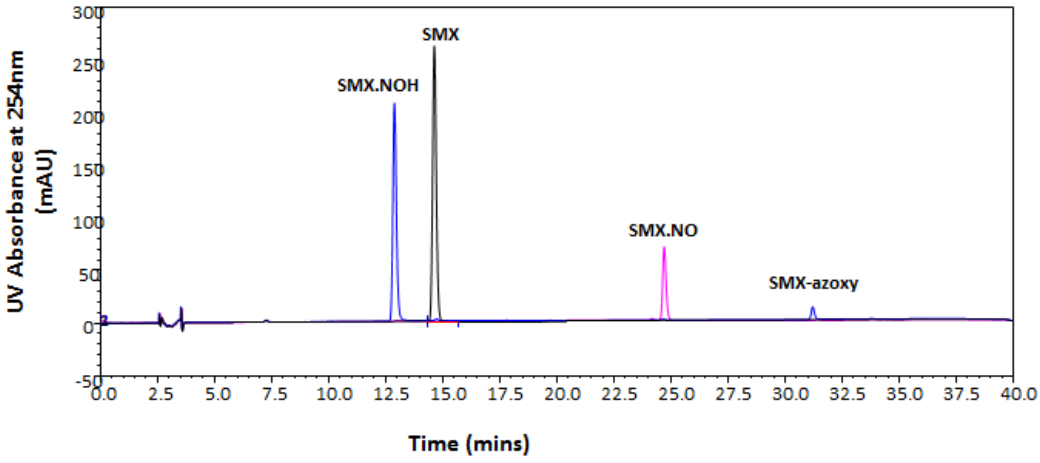


Figure 2.10 HPLC chromatogram showing for SMX.NOH, SMX and SMX.NO standards. Each standard (2 µM, 50 µL) was introduced into a HPLC tube and mixed thoroughly. The resulting mixture was then resolved using HPLC to determine retention times for each standard.

The retention times for SMX.NOH, SMX and SMX.NO were determined to be 12.8, 14.6 and 24.6 minutes respectively (figure 2.10). A peak with retention time of 31.1 minutes was suggestive of a dimerization product of SMX.NOH and SMX.NO as previously reported (Naisbitt et al. 2002). Furthermore, the ability of EBV-transformed B-cells, dendritic cells and HL60s to metabolise SMX was investigated. Both supernatants and whole cell lysates were analysed for SMX metabolites. Ascorbate (1 mM) was added to the culture medium to prevent auto-oxidation of SMX.NOH and thereby enhance its detection. SMX metabolites were not detected using HPLC method (figure 2.11).

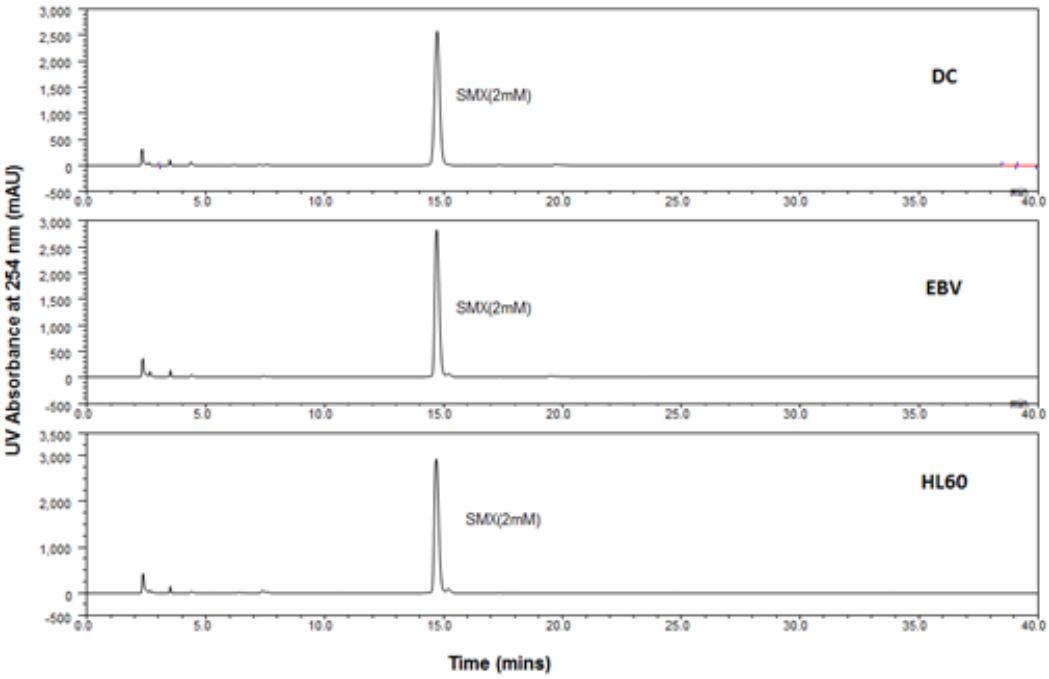


Figure 2.11 UV absorption spectrum to detect SMX metabolites in immune cells. EBV-transformed B-cells, DCs, orHL60s (2×10^6) were incubated in SMX (2 mM) for 16 hours and samples prepared for HPLC analysis as earlier described.

2.4.3 Proteomic analysis of drug metabolism enzyme in immune cells

Expressions of flavine-containing monooxygenases-1 and -3 by keratinocytes (Janmohamed et al. 2001; Vyas et al. 2006; Vyas et al. 2006; Sanderson et al. 2007) have been reported. However, there was no evidence of MPO expression in this cell type. Thus, we investigated SMX metabolism enzymes in other relevant immune cells (EBV-transformed B-cells). DCs have also been shown to express metabolic activity and metabolism of SMX has been demonstrated through the detection of covalently bound protein adducts (Sanderson et al. 2007). The purpose of these studies were to focus on metabolism and covalent binding in APC, not keratinocytes as these cells are directly involved in the priming of naïve T-cells in tissue draining lymph nodes.

Western blotting data suggested a differential expression of myeloperoxidase in EBV-transformed B-cells, dendritic cells and HL60 cells (figure 2.12A). FMO3 and thyroid peroxidase (TPO) were not detected (figure 2.12 B and C). Real time PCR was then used to verify the presence of messenger RNAs for MPO in immune cells (figure 2.13C). Messenger RNA for MPO was differentially expressed, HL60s > DCs > EBVs (figure 2.13C). Furthermore, mRNAs for FMO4, FMO5 and LPO were differentially expressed in all the immune cells (figure 2.13 A and B). MPO activity in the different immune cells was investigated using a peroxidation assay (figure 2.14A). Activity was detected in all cells with the following order of reactivity HL60 > DC > EBV. This was consistent with MPO protein expressions in the various cell types (figure 2.13C). Furthermore, the presence of hydrogen peroxide is important for MPO activity as ELISA data suggested SMX metabolism was significantly increased in the presence of hydrogen peroxide ($p = 0.006$; $n = 3$), see figure 2.14B.

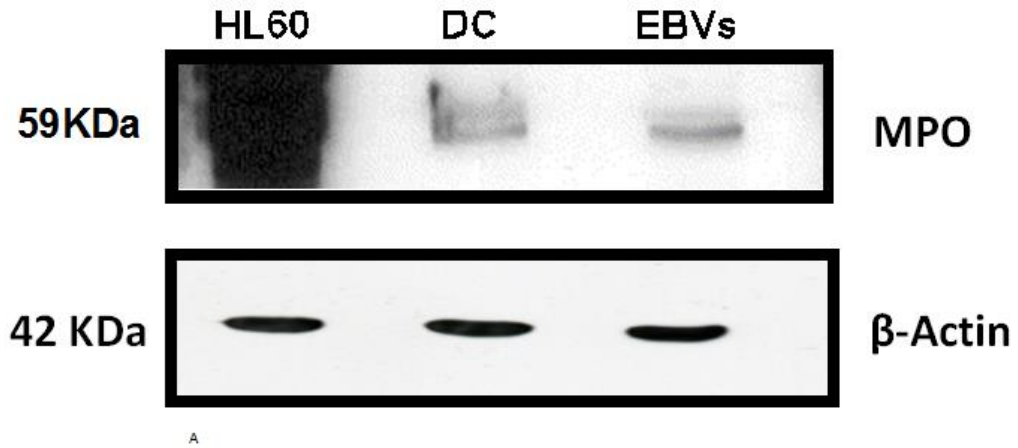


Figure 2.12 Western blotting for enzyme expression of SMX metabolism enzymes in immune cells. (A) MPO expression in immune cells. Cells (2×10^6/ml) were harvested and lysed according to method described earlier. Lysates were separated using 12% SDS-PAGE gel and blot probed for myeloperoxidase expression using an anti-MPO antibody (1:1000). β-Actin expression was used as the endogenous control. **(B)** FMO3 expression in EBV-transformed B-cell line and HL60 cell line using an anti-FMO3 antibody (1:1000), 10 µL recombinant FMO3 (250 µg/ml) as positive control. **(C)** Blot was probed for TPO using an anti-TPO antibody (1:1000) and using 10 µL recombinant TPO (250 µg/ml) as positive control.

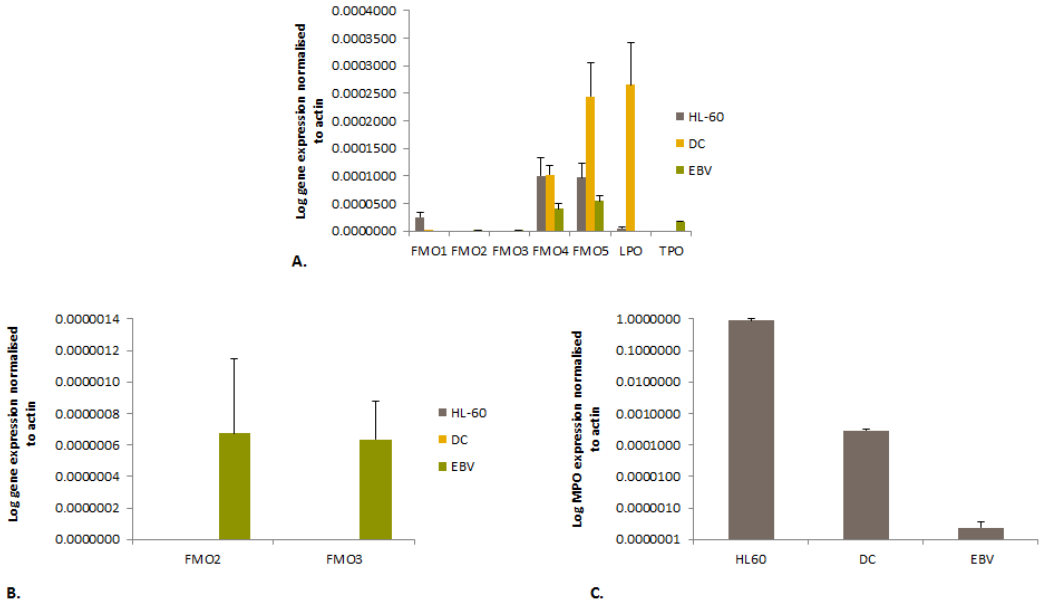


Figure 2.13 RT-PCR determination of mRNA for various FMOs and peroxidases expressed in HL60s, dendritic cells and EBV-transformed B-cells. (A)RNA extracts from HL60, dendritic cells and EBV-transformed B-cell lines were subjected to RT-PCR using specific primers for FMO1-5, LPO, TPO and β-actin (endogenous control). Relative mRNA levels were normalised to the corresponding β-actin mRNA expression. **(B)** RT-PCR for FMO2 and FMO3 expression in HL60, dendritic cells and EBV-transformed B cell line normalised to β-actin. **(C)** RT-PCR for MPO mRNA expression in HL60, dendritic cells and EBV- transformed B-cells.

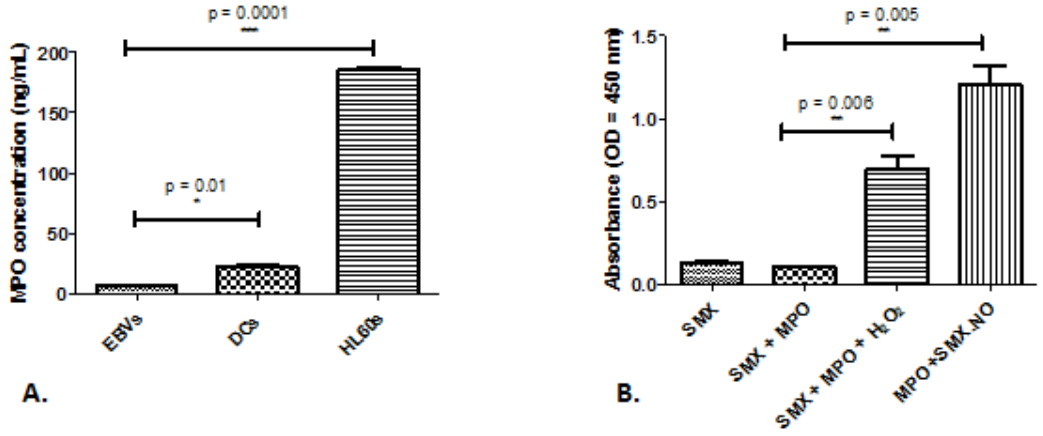


Figure 2.14 Peroxidation activity assay showing MPO concentration in HL60, DCs and EBV- transformed B-cell lines. **(A)** Clarified cell lysate samples (50 µL) and standard MPO (50 µL) were plated into a 96-well Microplate in duplicate. Equal volume of 2X Amplex Ultra Red reagents ™ working solution was added to all samples and standard MPO and wells developed according to manufacturer's instructions. **(B)** Confirmation of *in vitro* SMX metabolism using ELISA. SMX (2 mM) was incubated with MPO (100 µg/mL) with or without hydrogen peroxide (10 µM) for 1 hours. ELISA was then performed to determine level of SMX-protein adduct.

To further investigate MPO expression in immune cells LC-MS/MS analyses were employed. First, pure recombinant MPO was digested, processed and analysed by mass spectrometry. The protein was reduced with dithiothreitol (DTT) and alkylated with iodoacetamide prior to tryptic digestion. LC-MS/MS analysis was employed further to determine the expression of MPO in EBV-transformed B-cells using HL60s as a positive control.

Figure 2.15A illustrates the amino acid sequence of human MPO showing both heavy and light chains. The amino acid sequence of human MPO showing the various sites of natural modification is represented in figure 2.15B; notable is the presence of cysteine sulphenic acid modification on Cys316. The MS/MS spectrum of the peptide containing Cys316 is represented on figure 2.16. LC-MRM MS/MS analysis of recombinant MPO was performed to establish methods for determining the presence of MPO-derived peptides in EBV-transformed B-cells and HL60s.

Since MPO heavy and light chains would dissociate on SDS-PAGE gels, a series of bands were excised from Coomassie-Blue stained gels to ensure both chains were represented in MS data. Briefly, Coomassie-Blue staining of previously separated protein lysates obtained from HL60s, DCs or EBVs revealed multiple bands representing a variety of proteins with different molecular weights

(figure 2.17). Bands representing proteins migrating between 36 KDa and 90 KDa were excised and an in-gel tryptic digest performed to determine the presence of peptides consistent with MPO expression. The MPO sequence coverage for HL60 and EBVs were found to be 39.6% and 1.4% respectively (figure 2.18). The multiple reaction monitoring (MRM) of MPO peptides in HL60 cell lines and EBV-transformed B-cell line is illustrated in figure 2.19.

A.

Myeloperoxidase sp|P05164|PERM_HUMAN. Sequence coverage 71.4%

MGVPFFSSLRCMVDLGPCWAGGLTAEMKLLLALAGLLAILATPQPSEGAAPAVLGEVDTSLVLSSMEEAKQLVDKAY
KERRESIKQRLRSGSASPMELLSYFKQPVAATRTAVRAADYLHVALDLLERKLRSLWRRPFNVTDVLTPAQLNVLSK
SSGCAYQDVGVTCPEQDKYRTITGMCNNRRSPTLGASNRAFVRWLPAEYEDGFSLPYGWTPGVKRNGFPVALARAVS
NEIVRFPTDQLTPDQERSLMFMQWGQLLDHDLDFTPEPAARASFVTGVNCETSCVQQPPCFPLKIPPNDPRIKNQAD
CIPFFRSCPACPGSNITIRNQINALTSFVDASMVYGSEEPLARNLRNMSNQLGLLAVNQRFQDNGRALLPFDNLHDD
PCLLTNRSARIPCFLAGDTRSSEMPELTSMHTLLLREHNRLATELKSLNPRWDGERLYQEARKIVGAMVQIITYRDY
LPLVLGPTAMRKYLPTYRSYNDSVDPRIANVFTNAFRYGHTLIQPFMFRLDNRYQPMEPNPRVPLSRVFFASWRVVL
EGGIDPILRGLMATPAKLNRQNQIAVDEIRERLFEQVMRIGLDLPALNMQRSRDHGLPGYNAWRRFCGLPQPETVGQ
LGTVLRNLKLARKIMEQYGTPNNIDIWMGGVSEPLKRKGRVGPLLACIIGTQFRKLRDGDRFWWENEGVFSMQQRQA
LAQISLPRIICDNTGITTVSKNNIFMSNSYPRDFVNCSTLPALNLASWREAS

Signal peptide, **MPO light chain**, **MPO heavy chain**

B.

	Feature key	Position(s)	Length	Description	Graphical view
Molecule processing					
	Signal peptide	1 – 48	48	Ref.12 Ref.13	
	Chain	49 – 745	697	89 kDa myeloperoxidase	
	Chain	155 – 745	591	84 kDa myeloperoxidase	
	Chain	165 – 745	581	Myeloperoxidase	
	Chain	165 – 278	114	Myeloperoxidase light chain	
	Chain	279 – 745	467	Myeloperoxidase heavy chain	
Sites					
	Active site	261	1	Proton acceptor	
	Metal binding	262	1	Calcium	
	Metal binding	334	1	Calcium	
	Metal binding	336	1	Calcium; via carbonyl oxygen	
	Metal binding	338	1	Calcium	
	Metal binding	340	1	Calcium	
	Metal binding	502	1	Iron (heme axial ligand)	
	Binding site	260	1	Heme (covalent; via 3 links)	
	Binding site	408	1	Heme (covalent; via 3 links)	
	Binding site	409	1	Heme (covalent; via 3 links)	
	Site	405	1	Transition state stabilizer	
Amino acid modifications					
	Modified residue	316	1	Cysteine sulfenic acid (-SOH)	
	Glycosylation	139	1	N-linked (GlcNAc...) Ref.17	
	Glycosylation	323	1	N-linked (GlcNAc...) Ref.13 Ref.18	
	Glycosylation	355	1	N-linked (GlcNAc...) Ref.13 Ref.19	
	Glycosylation	391	1	N-linked (GlcNAc...) Ref.13 Ref.19	
	Glycosylation	483	1	N-linked (GlcNAc...) Ref.13 Ref.17 Ref.18	
	Glycosylation	729	1	N-linked (GlcNAc...) Ref.13	
	Disulfide bond	167 ↔ 180			
	Disulfide bond	281 ↔ 291			
	Disulfide bond	285 ↔ 309			
	Disulfide bond	319		Interchain	
	Disulfide bond	387 ↔ 398			
	Disulfide bond	606 ↔ 663			
	Disulfide bond	704 ↔ 730			

Figure 2.15 LC-MS/MS analysis of MPO. (A) Amino acid sequence of human recombinant myeloperoxidase showing MPO light chain and MPO heavy chain with sequence coverage of 71.4%. **(B)** Amino acid sequence of human MPO showing key features, positions, sites and amino acid modifications.

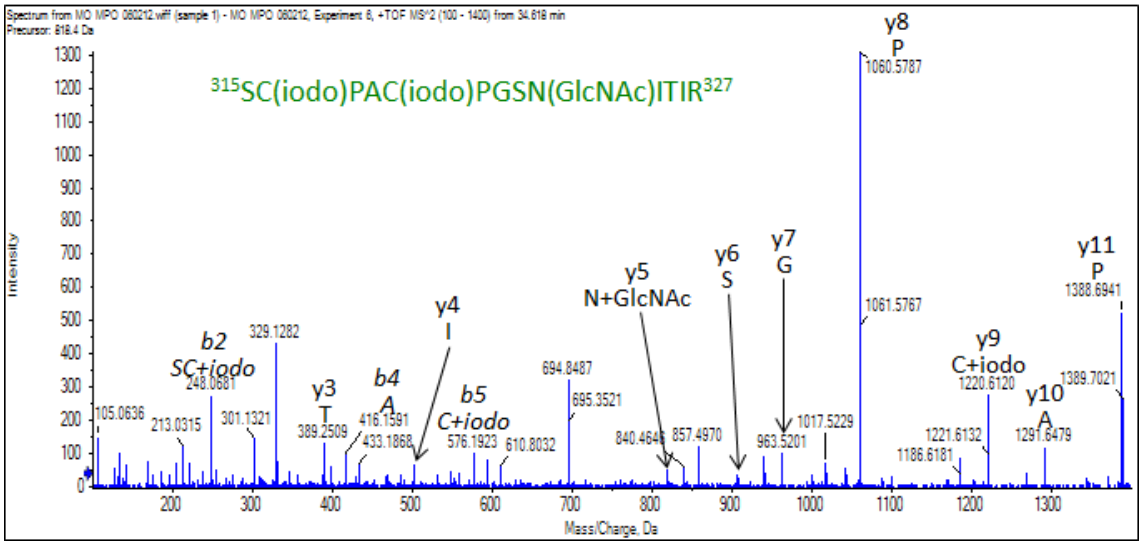


Figure 2.16 LC-MS/MS sequence analysis of MPO-derived peptide (315SCPACPGSNITIR327). MPO was reduced with dithiothreitol (DTT) and alkylated with iodoacetamide prior to tryptic digestion before LC-MS/MS analysis.

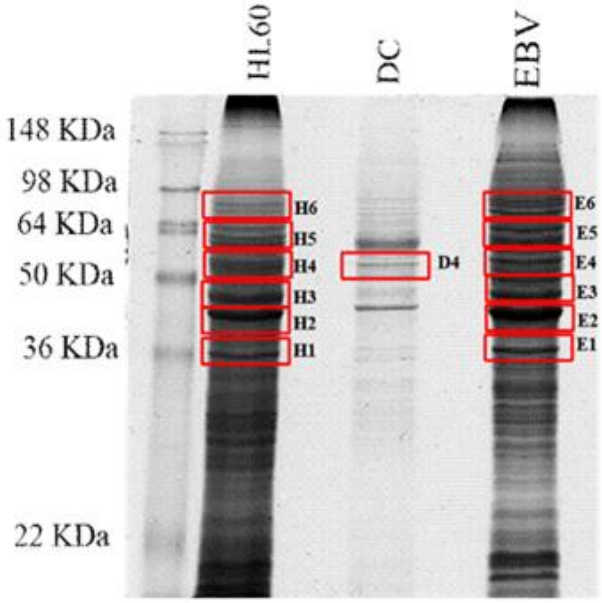


Figure 2.17 Coomassie Blue stain of proteins. Cells from HL60, DCs and EBV-transformed B-cells were lysed and separated by 12% SDS-PAGE followed by Coomassie blue staining to visualise proteins. Bands of interest were then excised followed by in-gel tryptic digest and LC-MS/MS analysis.

Figure 2.18 MPO detection in immune cells. HL60 and EBV-transformed B-cell proteins were electrophoresed on SDS-PAGE gels and a series of bands were excised corresponding to the approximate molecular weight of MPO. The proteins therein were digested with trypsin, extracted from the gel and analysed by LC-MS/MS or LC-MRM-MS/MS on a QTRAP5500 mass spectrometer. HL60 cells and EBV-transformed B-cell line showed peptides identified at > 95% (green) and > 90% (yellow) confidence limits respectively.

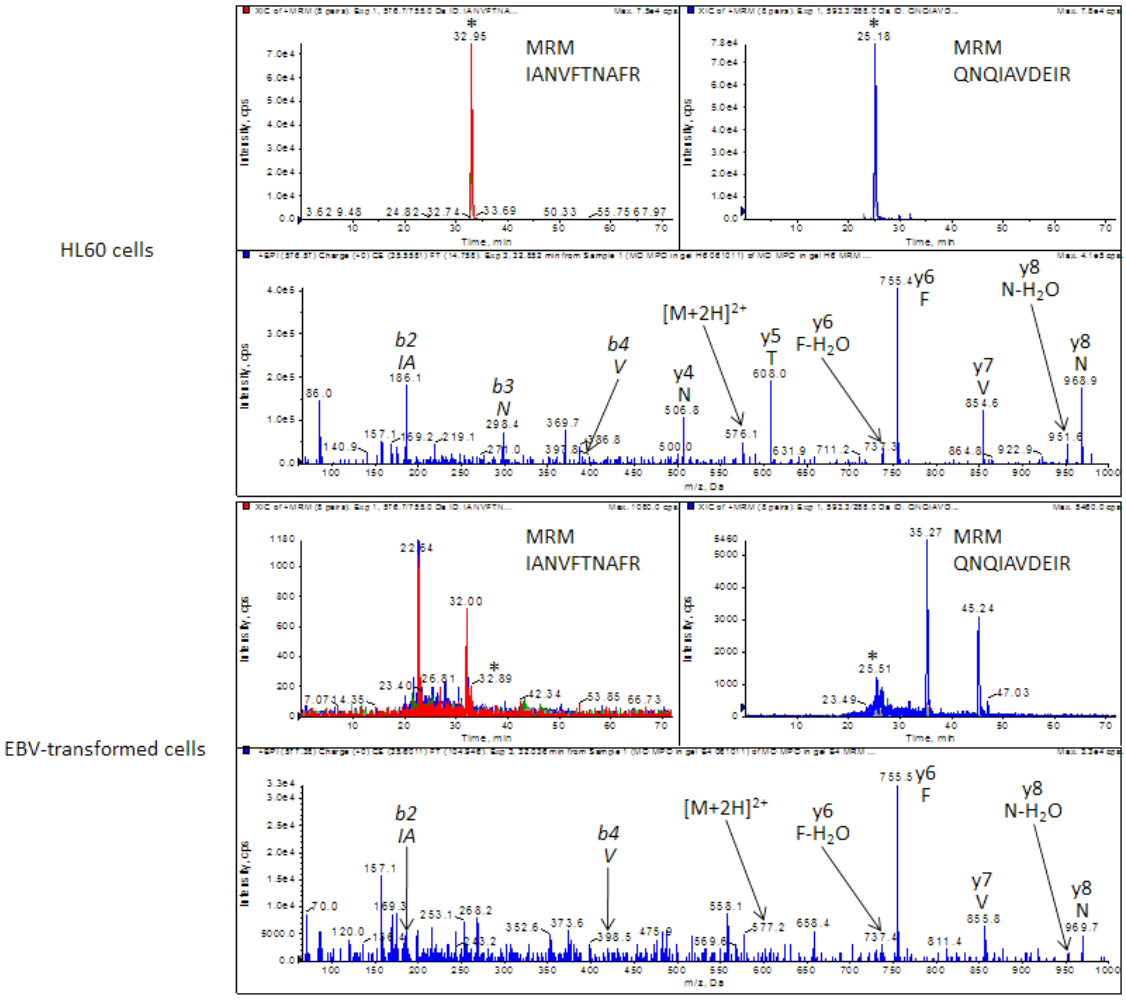


Figure 2.19 LC-MRM MS/MS analysis of MPO peptides in HL60 cells and EBVs using MRM for two distinct MPO peptides (IANVFTNAFR and QNQIAVDEIR).

Even with the MRM MS data obtained (figure 2.19) the evidence for the presence of MPO in EBV-transformed B-cells was very weak and probably would not stand on its own as sufficient to confirm the expression of the protein in these cells. A complete analysis of the whole cell lysates from EBVs using the TT5600 in discovery mode as illustrated in figure 2.20 revealed 2658 proteins but no evidence of MPO (figure 2.21). Of the identified proteins, 1090 exhibited catalytic activity and 80 were oxido-reductases. In contrast with the RNA data,

MPO, LPO, TPO and FMOs were not expressed at sufficient levels for MS detection.

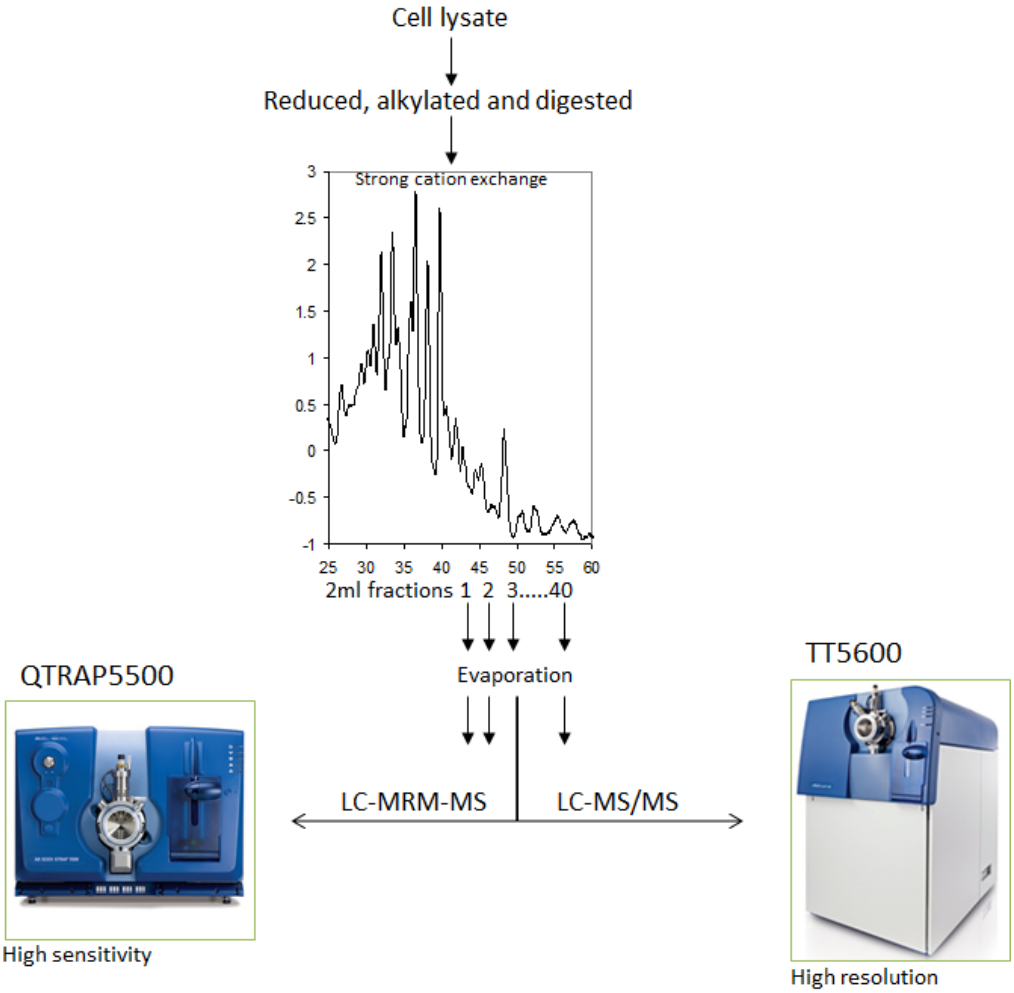


Figure 2.20 Schematic representation of steps involved LC-MRM and LC-MS/MS analysis of protein lysates. Cell lysis was performed and protein reduced, alkylated and digested and cation exchange performed on samples. Samples were then analysed using LC-MRM-MS and LC-MS/MS.

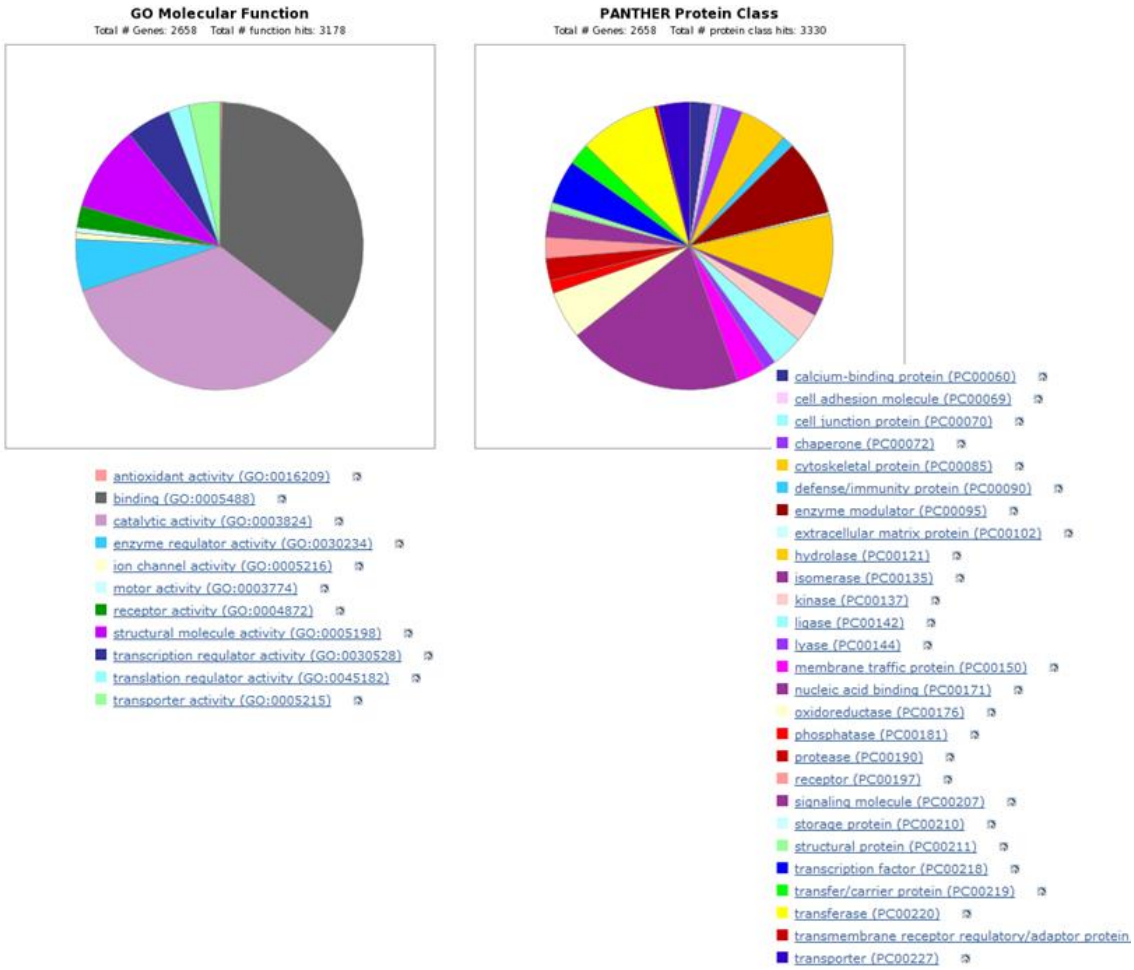


Figure 2.21 An analysis of the proteins using PANTHER (Protein Analysis Through Evolutionary Relationships, www.pantherdb.org) revealed a range of molecular functions and protein classes. 2658 proteins were identified using the TT5600 in discovery mode.

2.5 Discussion

The liver is the main organ for drug metabolism. Hepatic metabolism of SMX has been well characterized by a number of research groups (Cribb and Spielberg 1990; Cribb et al. 1993; Cribb et al. 1995; Sanderson et al. 2007). Normally, reactive metabolites generated in the liver are readily detoxified with the exception of certain pathologic conditions like HIV, neurodegenerative disorders, cancers and cystic fibrosis (Pirmohamed and Park 2001; Pirmohamed et al. 2002; Townsend et al. 2003). This is partly due to the high concentration of glutathione and glutathione transferase enzymes in the liver (Neil 1980; Coles et al. 2001; Wu et al. 2004).

The stability of reactive metabolites within the systemic circulation has been a subject of debate (Vyas et al. 2006; Vyas et al. 2006; Roychowdhury et al. 2007). CYP2C9 and MPO are critical human hepatic enzymes involved in SMX metabolism to its hydroxylamine metabolite, SMX.NOH (van der Ven et al. 1994; Cribb et al. 1995; Gill et al. 1996; Mitra et al. 1996; Pirmohamed and Park 2001; Park et al. 2005; Sanderson et al. 2007; Kagaya et al. 2012). SMX.NOH spontaneously reacts with readily available molecular oxygen to form SMX.NO (Cribb et al. 1991; Naisbitt et al. 1996). Due to its high reactivity and low stability, SMX.NO generally reacts with SMX.NOH to form a dimer (azo or axoxy dimers) or with proteins to generate antigenic epitopes for T cell receptor recognition (Cribb et al. 1991; Naisbitt et al. 1996; Manchanda et al. 2002; Naisbitt et al. 2002).

Chemically reactive drug metabolites have been implicated in a number of idiosyncratic drug reactions and the skin is the major target organ and immune

cells have been implicated (Park et al. 1998; Roychowdhury and Svensson 2005). Cutaneous reactions to SMX may present as mild skin rash to more complex, severe and sometimes life threatening reactions like SJS and TEN (van der Ven et al. 1991; Gruchalla et al. 1998; Mistry et al. 2009; Harr and French 2010; Taqi et al. 2012). Although the skin is highly vascularised and possess a wide surface, its drug metabolism capability is limited (Pannatier et al. 1978; Mukhtar and Bickers 1981; Baron and Merk 2001; Svensson 2009; Sharma and Uetrecht 2013). Cutaneous expressions of FMO1, FMO3 (Janmohamed et al. 2001; Vyas et al. 2006) and sulfotransferase (Windmill et al. 1998; Higashi et al. 2004) have been characterized in the human skin however, this is not the case with the cytochrome P450 superfamily (Sharma and Uetrecht 2013). Furthermore, high levels of MPO have been reported in dendritic cells (Pickl et al. 1996; Satthaporn and Eremin 2001; Sanderson et al. 2007) and HL60 cells (Meier et al. 1991; Hachiya et al. 2000; Kim et al. 2010).

In order to determine the role of localised metabolism in SMX hypersensitivity reactions, using immune cells we investigated the enzyme expression and activity *ex vivo*. The metabolism capacity of these APCs utilised in functional T-lymphocyte experiments was assessed using a battery of immunochemical assays (ELISA, Western blotting). The optimum time for SMX metabolism in EBV-transformed B-cells as detected by ELISA was determined to be 16 hours. There was a correlation between SMX-protein adduct formation observed when HL60 cells, dendritic cells or EBV-transformed B-cell lines were incubated with SMX for 16 hours and the levels of MPO expression as demonstrated by RT-PCR and Western blotting data. RT-PCR was used to investigate the relative expression of messenger RNAs for MPO and other drug metabolism enzymes

(FMO 1-5 and TPO) that may be involved in SMX metabolism. MPO protein expression was highest in HL60 cells followed by dendritic cells and EBV-transformed B-cells.

Peroxidation assay revealed differential MPO activity in HL60 cells > DCs > EBV-transformed B-cells. This is consistent with data from both mRNA expression and protein expression studies in the respective cell types. MPO requires the presence of hydrogen peroxide for its enzymatic activity (Iwamoto et al. 1987; Pulli et al. 2013). Correspondingly, SMX.NO binding to purified MPO was found to be statistically higher in the presence of hydrogen peroxide. Although EBV-transformed B-cells expressed mRNA for FMO2 and FMO3, the protein for both enzymes was not detected using Western blotting. It is possible that protein translation does not take places or protein is degraded shortly after translation. The expression of FMO1 and FMO3 has been reported for keratinocytes (Janmohamed et al. 2001; Vyas et al. 2006). The authors demonstrated that FMO3 was essential for SMX metabolism in keratinocytes. In humans, FMO2 is involved in N-oxidation of some primary alkylamines but the FMO2 gene also encodes for a truncated version of the enzyme that is devoid of catalytic activity (Dolphin et al. 1998). Hence, it is unlikely that the SMX metabolism observed in EBV-transformed B-cells was a result of FMO3.

SMX.NO-specific T-cell clones were generated and used to delineate the role of APC metabolism in T-lymphocyte activation. These TCCs were not activated with SMX, the parent drug (chapter 3). APCs pulsed with SMX for 16 hours were found to stimulate SMX.NO-specific TCCs, suggesting that SMX metabolism occurs in APCs in sufficient quantities to generate T-cell antigens. Furthermore,

glutaraldehyde fixation of APCs and methimazole inhibition of enzymatic activity in APCs significantly decreased T-lymphocyte proliferation when both APCs and SMX.NO-specific T-lymphocytes were incubated with SMX for 48 hours. Methimazole is a non-selective inhibitor of the peroxidases reported to inhibit SMX metabolism.

Tracking SMX metabolites in APC using HPLC method was unsuccessful. The inability to detect SMX metabolites in immune cells may be due the intrinsically reactive nature of SMX.NO resulting in spontaneous protein haptenation (Naisbitt et al. 1996; Cheng et al. 2008). These data emphasis that immunochemical detection of SMX adducts is more sensitive than direct analysis of SMX metabolism. In order to aid the identification of MPO in cell lysates, mass spectrometric analysis of recombinant human MPO was performed. MPO consists of 745 amino acid residues with 17 cysteine residues. SMX.NO has previously been shown to bind and covalently modify cysteine residues in human serum albumin (Cheng et al. 2008; Callan et al. 2009; Lavergne et al. 2009). Cys316 is present in vivo as the sulfenic acid whereas Cys319 forms an interchain disulphide. These cysteine residues are likely to play critical roles in SMX-MPO adduct formation. The details of SMX.NO-MPO modification will be discussed in chapter 6. HL60 and EBV-transformed whole cell proteins were electrophoresed on SDS-PAGE gels and a series of bands were excised corresponding to the approximate molecular weight of MPO. The proteins therein were digested with trypsin, extracted from the gel and analysed by LC-MS/MS or LC-MRM-MS/MS on a QTRAP5500 mass spectrometer. Analysis in multiple reaction monitoring (MRM) mode affords a

10-100X increase in sensitivity over conventional LC-MS on the same instrument.

Two peptides were chosen to design the MRM transitions. Transitions were comprised of two masses as a uniquely identifying pair of ions for each of the target peptides. The first mass was the mass/charge (m/z) ratio of the parent peptide. For IANVFTNAFR, the m/z of the doubly charged parent ion was 576.6. This parent ion mass was then paired with the dominant fragment ion masses of 608, 755, 854 and 968. Thus the four transitions for detection of IANVFTNAFR were 576.6/608, 576.6/755, 576.6/854 and 576.6/968. Each time the mass spectrometer detected a parent ion m/z of 576.6 in combination with a fragment ion mass of 608, 755, 854 or 968, a full-spectrum MS/MS was triggered to confirm the identity of the peptide. At the same time, the extracted ion counts (XIC) for the combined transitions provided a measure of the level of that peptide in the sample.

Even with MRM MS, the evidence for the presence of MPO in EBV-transformed cells was very weak and probably would not stand on its own as sufficient to confirm the expression of MPO in EBV-transformed B-cells. Therefore, another approach was attempted. The cells were lysed and the resulting protein mixtures were reduced, alkylated and digested with trypsin. The highly complex mixture of peptides was then simplified by pre-fractionation using strong cation exchange chromatography, with the eluted peptides being collected as series of fractions. The fractions were analysed individually by LC-MRM-MS on the QTRAP as before, and also by discovery LC-MS/MS on a high speed/high resolution mass spectrometer (Triple TOF 5600).

This combined approach would provide the highest probability of detecting MPO in EBV-transformed cells. MPO was not detected on the QTRAP even when using MRM. The number of proteins identified using the TT5600 in discovery mode was 2658. An analysis of the proteins using PANTHER (Protein Analysis Through Evolutionary Relationships, www.pantherdb.org) revealed a range of molecular functions and protein classes that might reasonably be expected to include MPO, for example catalytic activity and oxidoreductase. However, MPO was not identified in this analysis. Thus, the mass spectrometric evidence for MPO in EBV-transformed-cells was equivocal. This means that its level of expression was simply too low to be detected using some of the most sensitive analytical instrumentation available. In conclusion, despite the lack of conclusive MS evidence for the presence of MPO in EBV-transformed B-cells, other evidence (RT-PCR and Western blotting) suggested low levels of MPO expression in EBV-transformed B-cell is involved in generating antigenic determinants that activate T-cells.

Chapter 3: HLA-restricted activation of nitroso-sulphamethoxazole-specific CD4 positive T-lymphocytes

Contents

3.1 Introduction

A number of drug hypersensitivity reactions are associated with the expression of the cell surface glycoproteins called human leucocyte antigens, HLAs (Mallal et al. 2002; Chen et al. 2011). HLAs play a critical role in immunity and disease and represent the loci of genes that encode for the major histocompatibility complex (MHC) in humans. The high degree of polymorphism in the HLA allele is critical for immune surveillance as each variant of a particular HLA molecule interacts with different peptides. Hence HLAs are essential in both immune mediated drug hypersensitivity reactions and induction of self-tolerance (Yun et al. 2012). A number of HLA alleles provide protection against a variety of diseases (Temajo and Howard 2009; Han et al. 2012), while others serve as risk or predisposing factors for autoimmune diseases including drug hypersensitivity (Mallal et al. 2002; Caillat-Zucman 2009; Kim et al. 2010; Chen et al. 2011). The MHC region on chromosome 6 has been extensively researched and reported to contain about 100 genes that regulate immune function and encode cell surface antigen presenting molecules (Beck et al. 1999; Mungall et al. 2003; Boulanger and Shatz 2004; Torres et al. 2012).

Based on molecular structure and function, the MHC complex is broadly classified into two major classes, MHC I and MHC II; both consisting of α- and β-domains (molecules are membrane-bond heterodimers). MHC I complexes result from a non-covalent interaction between a heavy polypeptide chain (44 kDa) and a light β_2-microglobulin (12 kDa) and are expressed on all nucleated cells. On the other hand, MHC II is composed of glycoproteins which consist of α and β polypeptide molecules weighing 34 kDa and 29 kDa, respectively. The α_1 and α_2 domains of MHC I and MHC II interact with both ends of a β-plate sheet to

form the peptide binding cleft (groove). The peptide-binding groove of MHC I accommodates peptides of between 8 and 11 amino acid residues and shows restriction at both ends (Speir et al. 2001).

In humans, the three sub-classes of MHC I gene includes HLA-A, HLA-B and HLA-C while the three variants of MHC II genes are HLA-DQ, HLA-DP and HLA-DR. The peptide binding groove of MHC II is open at both ends and capable of accommodating longer peptides, 12-25 amino acid residues (Rammensee 1995).

HLAs are often referred to as antigen display molecules and they are involved in T-lymphocyte activation. T-lymphocytes are only able to recognise antigens that are bound to MHC molecules. MHC molecules interact with short peptide fragments generated from intracellular (MHC I) or from extracellular (MHC II) proteins and present these antigens to $CD8^+$ and $CD4^+$ T-lymphocytes, respectively. Peptides associated with specific HLA molecules migrate to the cell surface for antigen presentation resulting in the secretion of multiple cytokines and cytotoxic molecules that mediate hypersensitivity reactions.

HLA nomenclature is controlled by the WHO Nomenclature Committee for Factors of the HLA System as illustrated in figure 3.1 below. Each HLA allele has a particular number comprising 4 sets of digits separated by colons.

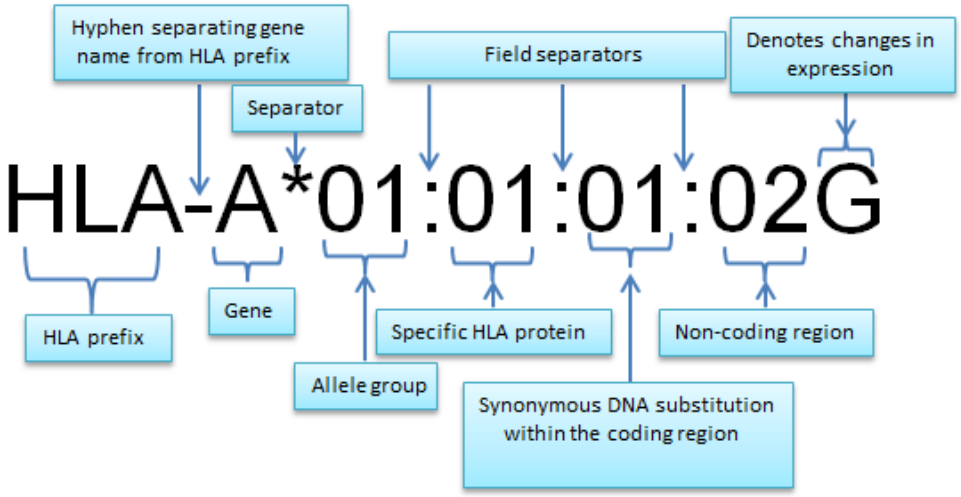


Figure 3.1- Schematic illustration of the nomenclature of an HLA allele.

The molecular mechanisms involved in HLA-associated hypersensitivity reactions are complex and often dependent on a unique interaction between the drug molecule and the HLA allele implicated. The association between abacavir hypersensitivity syndrome and HLA-B*57:01 was first reported in 2002 (Hetherington et al. 2002; Mallal et al. 2002). The exact molecular mechanism of abacavir and HLA-B*57:01 interaction only became clear about a decade later (Chessman et al. 2008; Adam et al. 2012; Illing et al. 2012; Norcross et al. 2012; Ostrov et al. 2012). The so called "altered self-peptide repertoire model" is a relatively new concept that explains the fundamental basis of abacavir hypersensitivity syndrome. The interaction between abacavir and the F-pocket of the MHC binding grove of HLA-B*57:01 alters the repertoires of self-peptides that bind to HLA-B*57:01 and are eventually presented to TCRs resulting in an immune response (Adam et al. 2012). Asp 114 and Ser 116 are specific amino acid residues known to facilitate the interaction between abacavir and the F-pocket of the MHC binding grove (Chessman et al. 2008). Pre-prescription screening of susceptible patients has been shown to significantly reduce the

occurrence of abacavir hypersensitivity and this illustrates the concept of personalised medicine (Hughes et al. 2004; Zucman et al. 2007; Mallal et al. 2008; Bharadwaj et al. 2010; Chen et al. 2011).

HLA-B*15:02 is associated with carbamazepine-induced SJS/TEN in both the Han Chinese and the Thai populations (Chung et al. 2004; Alfirevic et al. 2006; Hung et al. 2006; Lonjou et al. 2006; Locharernkul et al. 2008). Carbamazepine interacts directly with HLA-B*15:02 or an embedded HLA-B*15:02 binding peptide to activate T-cells in these patients (Wei et al. 2012). Interestingly, HLA-B*15:02 is only associated with carbamazepine-induced SJS/TEN but not with maculopapular exanthema or drug reactions with systemic symptoms, DRESS (Hung et al. 2006). In Caucasian and Japanese populations, HLA-A*31:01 is the susceptible allele implicated in carbamazepine-induced hypersensitivity reaction (McCormack et al. 2011; Ozeki et al. 2011). The drug specific $CD8^+$ T-cell response in certain HLA-A*31:01 positive patients has been shown to be HLA-A*31:01 restricted (Lichtenfels et al. 2014). However, $CD4^+$ T-cells have been isolated from the same patient and the drug is presented in the context of various HLA class II molecules.

A strong association between flucloxacillin-induced hepatic injury and HLA-B*57:01 was first described in 2009 (Daly et al. 2009). Regardless of this strong association, only 1 in 1000 individuals expressing the HLA-B*57:01 allele develops drug-induced liver injury (DILI). Unlike abacavir, flucloxacillin does not alter the repertoire of self-peptides binding to HLA-B*57:01 but covalently modifies endogenous protein which is processed and presented as antigenic determinants in the context of MHC molecules to drug specific T-cells (Norcross

et al. 2012; Monshi et al. 2013). Many other HLA-drug hypersensitivity/DILI associations have been described (see table 3.1); however, for the most part, the HLA-restriction of drug specific TCCs response has not been studied.

Table 3.1- Examples of HLA associated drug hypersensitivity reactions

Drug	ADR	HLA association	Reference	Drug-specific T-cells	Preferential drug presentation by the risk HLA	Reference
Aminopenicillins	HSS	A2, DRw52	Romano et al., 1998	+	_	_
Aspirin	Asthma	DPB1*03:01	Dekker et al., 1997	_	_	_
Hydralazine	Systemic Lupus	DR4	Batchelor et al., 1980	_	_	_
Lapatinib	DILI	DQA1*02:01 /DRB1*07:01	Spraggs et al., 2011	_	_	_
Lumiracoxib	DILI	DRB1*15:01/ DQB106:02 DRB5*01:01/ DQA1*01:01	Singer et al., 2010	_	_	
Nevirapine	Cutaneous reactions	Cw4 B*35:05	Likanonsakul et al., 2009 Chantarangsu et al., 2009	+	_	_
SMX	SJS/TEN	_	Pirmohamed, 2006	+	_	_
Ximelagatran	DILI	DRB1*07-DQA1*02	Kindmark et al., 2008	_	_	_
Abacavir	AHS	B*57:01	Mallal et al., 2002	+	+	Chessman et al., 2008
Allopurinol	SJS/TEN/HSS	B*58:01	Hung et al., 2005	+	+	Yun et al., 2013
Carbamazepine	SJS/TEN	B*15:02 in Asians A*31:01 in Japanese A*31:01 in Europeans	Chung et al., 2004 Ozeki et al.,2011 McCormack et al., 2011.	+	+	Chen et al., 2011
Flucloxacillin	DILI	B*57:01	Daly et al., 2009	+	+	Monshi et al., 2013
ASH-Abacavir hypersensitivity syndrome; SJS-Steven Johnson syndrome; TEN-Toxic epidermal necrolysis; HSS-Hypersensitivity syndrome; DILI-drug induced liver injury						

Very weak HLA-associations involving HLA-A29, -B12 and -DR7 alleles and SMX-induced cutaneous hypersensitivity reactions were previously documented in Caucasians (Roujeau et al. 1986), however, more recent work suggests there is no HLA-association with SMX-hypersensitivity (Pirmohamed 2006). Two distinct pathways of SMX recognition by drug responsive T-lymphocytes have been characterised. The first pathway is a metabolism–independent, non-covalent interaction between SMX and HLA molecules (Schnyder et al. 2000). The second pathway involves metabolism of SMX to a protein-reactive metabolite, nitroso-sulfamethoxazole which binds to cysteine residues on proteins to generate antigenic epitopes for T-lymphocytes (Schnyder et al. 2000; Farrell et al. 2003; Castrejon et al. 2010; Elsheikh et al. 2010).

3.2 Aims

It is assumed that the extensive modification of multiple proteins will generate peptide antigen for almost if not all HLA molecules; however, the extent of HLA restriction for SMX.NO-responsive T-cells is not known. We hypothesised that HLA allele may play an important role in the activation of SMX.NO-specific $CD4^+$ TCCs. The specific aims of this chapter were

- To generate SMX.NO responsive TCCs from SMX-hypersensitive patients with cystic fibrosis (CF).
- To define HLA molecules that present haptenic determinants to SMX.NO specific TCCs.
- To determine the extent of alloreactivity.
- To analyse TCR V_β expression on SMX-NO responsive TCCs.

3.3 Methods

3.3.1 Chemicals and reagents

CD4-APC, CD8-PE, anti-human HLA-ABC-PE and FITC mouse anti-human HLA-DR, DP and DQ monoclonal antibodies were purchased from BD Biosciences, Oxford, UK. TCR V_β repertoire kit was purchased from Beckman (Marseille, France). Interferon-γ, interleukin-13, interleukin-5, granzyme-B and perforin ELISpot kits including antibodies and substrate solution were purchased from Mabtech (Stockholm, Sweden). DNA extraction kits were purchased from Promega (Madison, USA). Purified NA/LE mouse anti-human HLA-ABC, HLA-DR, -DP, -DQ monoclonal antibodies were purchased from BD Bioscience (Oxford, UK). Mouse anti-human HLA-DP monoclonal antibody was obtained from AbD SeroTec (Kidlington, UK). Pico Green DNA assay kit was bought from Invitrogen (Paisley, UK).

3.3.2 Cell culture medium

Culture medium for T-cell comprised of RPMI 1640 supplemented with 10% human AB serum, 25 mM HEPES, 1000U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine and 25 μg/ml transferrin from Sigma-Aldrich (Dorset, UK). The culture media for DCs and EBV-transformed B-cells have been described in section 2.3.2.

3.3.3 Isolation of PBMCs

Venous blood (50 ml) was collected from four SMX-hypersensitive patients for cloning. Clinical features of the ADRs are described in table 3.2. Approval for the study was acquired from the Liverpool Local Research Ethics Committee and informed written consent was obtained. Peripheral blood mononuclear cells

(PBMCs) were isolated from patients' blood as previously described in section 2.3.3.

3.3.4 Lymphocyte transformation test (LTT)

LTT was performed on PBMCs isolated from SMX-hypersensitive patients using an established protocol (Nyfeler and Pichler 1997). Briefly, PBMCs (1.5×10^5 cells, 100 µL) were cultured with either 100 µL SMX (0.25-2 mM) or SMX.NO (10-80 µM) in triplicate wells in a 96-well U-bottom plate and incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 5 days. Tetanus toxin (5 µg/ml) and culture medium were used as positive and negative controls, respectively. [^{3}H]-thymidine (0.5 µCi/well) was added for the final 16 hours of incubation and lymphocyte proliferation was assessed as counts per minute (c.p.m) using liquid scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK). Proliferative responses were calculated as stimulation index (SI) = cpm in drug treated cultures/cpm in medium control. An SI ≥2 was considered a positive response.

3.3.5 Generation of drug-specific T-cell clones

For the separation of SMX and SMX.NO-specific T-cell clones (TCCs), PBMCs ($1x10^6$/ml) from hypersensitive patients were cultured with either SMX (2 mM) or SMX.NO (25 µM). On days 5 and 9, culture medium was supplemented with IL-2 (200 IU/ml) to expand the number of antigen-specific T-cells prior to cloning on day 14. Autologous Epstein-Barr virus (EBV)-transformed B-cell lines were used as antigen presenting cells in assays involving TCCs.

Antigen-specificity was assessed by culturing irradiated EBV-transformed B-cells ($1x10^4$/well) and SMX or SMX.NO with clones ($5x10^4$/well; 200 µl) for 48

hours. Proliferation was measured by the addition of [^{3}H]-thymidine followed by scintillation counting. Clones with a stimulation index of greater than 2 were expanded by repetitive stimulation with irradiated allogeneic PBMCs ($5x10^5$/well), IL-2 (5 µg/ml) and PHA (10 µg/ml). Dose-dependent proliferative responses to SMX.NO (5-50 µM) and SMX (0.25-2 mM) and the profile of secreted cytokines (IFN-γ, IL-5, IL-13 and granzyme-B ELISpot) were then measured.

Table 3.2- Clinical details of SMX-hypersensitive patients and the origin, phenotype and specificity of the T-cell clones

Patient ID	Clinical details (n)	Clones tested (n)	SMX-NO specific clone	CD Phenotype (%)		Proliferation (cpm)	
				CD4+	CD8+	Control	SMX-NO(50uM)
1:Female, Age 30	Maculopapular rash day 2 of treatment; 7 years since reaction	336	20	96.6	3.3	6840 ± 7406	14837 ± 13224
2: Male, Age 23	Maculopapular rash day 10 of treatment; 20 years since reaction	152	12	100	_	1849 ± 1659	5086 ± 3649
3: Female, Age 34	Maculopapular rash day 6 of treatment; 8 years since reaction	216	5	100	_	1058 ± 203	2740 ± 469
4: Female, Age 26	Periorbital oedema day 3 of treatment; 6 years since reaction	240	2	100	_	1584 ± 493	7613 ± 5294

3.3.6 Generation of EBV-transformed B-cells

PBMCs were transformed into B-cell lines using supernatant from the virus-producing cell line B9.58; Epstein-Barr virus (EBV) as previously described in section 2.3.5.

3.3.7 DNA extraction, quantification and HLA genotyping

PBMCs (5×10^6 cells) were resuspended in HBSS (200 µL) and added to aliquots of proteinase K (PK) solution dispensed into 1.5 ml microcentrifuge tubes. Cell lysis buffer (200 µL) was then added to each tube, followed by vortexing for 10 seconds. Tubes were incubated at 56°C for 10 minutes. 250 µL of binding buffer was added to each tube and vortexed for 10 seconds. Next, the content of each tube was transferred to a ReliaPrep binding column placed in an empty collection tube. Tubes were then centrifuged at 13,000 g for 1 minute in a bench top centrifuge making sure that the lysate had completely passed through the column. The flowthroughs were discarded and binding columns placed in fresh collection tubes. Column wash solution (500 µL) was added to each column and centrifuged at 13,000 g for 3 minutes and the flowthrough discarded. After washing, nuclease-free water (50 µL) was added to the column. Subsequently, the columns were centrifuged for 1 minute at 13,000 g to elute DNA (25-30% yield).

DNA concentration was quantified using the PicoGreen method. Briefly, 1×TE buffer was prepared according to manufacturer's instruction. Dilutions for a standard curve were generated using a DNA standard (1 ng/ml-1000 ng/ml). DNA standard (100 µL) was pipetted in duplicate into a 96-well plate. For samples measuring < 200 ng/µL on the nanodrop, a 1:10 dilution was performed while those measuring > 200 ng/µL were diluted 1:100. Samples (100 µL) were plated out in duplicate. PicoGreen working reagent was added to both standard DNA wells and wells containing samples under reduced light conditions. The plate was incubated for 5 minutes in the dark at room temperature. Sample fluorescence was then measured using a Beckman Coulter

DTX880 Microplate reader. DNA concentrations were then determined using a standard template for the PicoGreen assay. DNA samples (200 ng/μL) were then sent to Histogenetics (New York, USA) for high-resolution HLA sequence-based genotyping.

3.3.8 Flow cytometry

T-lymphocytes were stained using fluorescent antibodies (see below) and cells acquired using a FACS Canto II (BD Biosciences). Data was analyzed by Cyflogic (http://www.cyflogic.com/). A minimum of 50,000 lymphocytes were acquired using forward scatter and side scatter characteristics.

3.3.8.1 T-cell phenotyping

TCCs were examined for CD4 and CD8 cell surface expression. Cells were acquired using flow cytometry as described above. Briefly, T-cell suspensions (100 μL) were stained with CD4-APC (3 μL) and CD8-PE (3 μL) antibodies and incubated at 4°C for 20 minutes in the dark. Cells were then washed and resuspended in 200 μL of FACS buffer before analysis for CD4 and CD8 cell surface expression.

3.3.8.2 Determination of MHC I and II expression on T-cell clones

MHC I and MHC II expressions on some SMX.NO-specific TCCs were measured using flow cytometry. T-cell suspensions (100 μL) from various TCCs were stained with MHC I-PE and MHC II-FITC (3 μL each) antibodies and incubated at 4°C for 20 minutes in the dark. Cells were then washed and resuspended in 200 μL of FACS buffer before analysis.

3.3.9 Dose-response and cross reactivity assay

SMX.NO specific TCCs (5×10^4 cells, 50 µL) were co-cultured with irradiated autologous EBV-transformed B-cells (1×10^4 cells, 50 µL) in the presence of either SMX (1-2 mM) or SMX.NO (5-50 µM) in duplicate in a 96-well U-bottom plate. Plates were incubated under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 hours of the incubation and T-lymphocyte proliferation evaluated using scintillation counting.

3.3.10 ELISpot assay

ELISpot plates were coated with 100 µL/well of interferon gamma capture antibody (15 µg/ml) and incubated overnight at 4°C. Wells were washed five times with sterile PBS and then blocked with 200 µL of T-lymphocyte culture medium for 30 minutes at room temperature. Drug specific TCCs (5×10^4, 50 µL) were added to wells along with autologous irradiated EBV-transformed B-cells (1×10^4, 50µL). In other experiments, IFN-ϒ secretion from PBMCs ($0.5\text{-}1.0\times10^5$) isolated from SMX-hypersensitive patients was analysed.

Cells were cultured in the presence or absence of SMX.NO (50 µM, 100 µL) and plates incubated at 37˚C under an atmosphere of 95% O_2/5% CO_2 48 hours. Cells were discarded after 48 hours and wells washed five times with 200 µL PBS. Biotin-labelled detection antibody was diluted to 1 µg/ml in PBS containing 0.5% FBS and 100 µL added to the wells. Plates were incubated at room temperature for 2 hours at room temperature. Wells were then washed five times with PBS. 100 µL/well streptavidin-ALP diluted in PBS containing 0.5% FBS (1:1000) was added to wells and incubated for 1 hour at room

temperature. Wells were washed five times with PBS (200 µL) and BCIP/NBT substrate (100 µL/well) was added for 15 minutes at room temperature in the dark. Wells were inspected for the development of spots and then washed under slow running tap water. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader (Cadama Medical, Stourbridge, UK).

3.3.11 MHC restriction assay

Anti-human HLA-A, -B, -C (MHC I), and anti-human HLA-DP, -DQ, -DR (MHC II) antibodies (5 µg/mL) were used to determine whether SMX.NO presentation to drug- specific TCCs was MHC class I/II restricted. Autologous EBV-transformed B-cell lines (1×10^4, 50 µL) were pre-incubated with either MHC I or MHC II blocking anti-bodies (5 µg/ml) at 37°C under an atmosphere of 95% O_2/5% CO_2 for 30 minutes. The APCs were then co-cultured with SMX.NO specific TCCs (5×10^4, 50 µL) with or without SMX.NO (50 µM) for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting. Cytokine secretion profiles (IFN-ϒ, IL-5, IL-13and granzyme-B) were also determined following MHC I and MHC II block using ELISpot analysis. Similar MHC restriction assay was also performed for the subclasses of MHC II (HLA-DP, -DQ and –DR).

3.3.12 T-cell receptor V_β expression

Ten tubes (1-10) were required for TCR V_β typing of individual clones. T-cell suspensions (50 µL) were pipetted into each tube. Anti-CD3 antibody (3µL) was introduced into tubes 2-10. TCR Vβ antibodies (5 µL) labelled A-H were then introduced into tubes 3-10 containing TCCs + anti CD3 antibody. Each TCR Vβ

antibody cocktail was used to investigate three TCRs, twenty-four in total. Tube 1 had no antibody and was used to gate the T-lymphocyte population during flow cytometry. Tubes were incubated at room temperature for 20 minutes. Unbound antibodies were washed with FACS buffer (1 ml), 1500 rpm for 5 minutes at room temperature. Finally, TCCs were resuspended in FACS buffer (200 µL) and samples analysed.

3.3.13 APC mismatch assay (T-cell proliferation and ELISpot)

SMX.NO-specific T-cell clones (5×10^4 cells) generated from two SMX-hypersensitive patients were co-cultured with either autologous or heterologous EBV-transformed B-cells (1×10^4 cells) with or without SMX.NO (50 µM) in a 96-well U-bottom plate. The selection of heterologous APCs was based on the expression of either HLA-DQB1*05:01:01G/ DQB1*06:03:01G or DQB1*02:01:01G/DQB1*02:01:01G expressed by patient 1 and patient 2 (see table 3.3; page 129). Plates were incubated at 37°C, 5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi) added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting. Cytokine secretion profile (IFN-ϒ, IL-5, IL-13 and granzyme-B) was also determined using ELISpot analysis.

3.3.14 Statistical analysis

Mean values and standard deviations were calculated, and statistical analysis was performed using paired T tests (Sigma plot 12 software).

3.4 Results

3.4.1 Lymphocyte transformation test and IFN-ϒ secretion

Lymphocytes from SMX-hypersensitive patients with CF were stimulated *in vitro* with graded concentrations of SMX (0.25-2 mM) and SMX.NO (10-80 μM). Lymphocytes from patients 1 and 2 proliferated weakly in response to SMX. The LTT result to SMX in patients 3 and 4 were negative (SI <2). Lymphocytes from 2 out of the 4 patients proliferated in response to SMX.NO (figure 3.2 A). IFN-ϒ secretion by PBMCs in response to graded concentrations of either SMX or SMX.NO was either weak or non-existent for all the patients (figure 3.2 B).

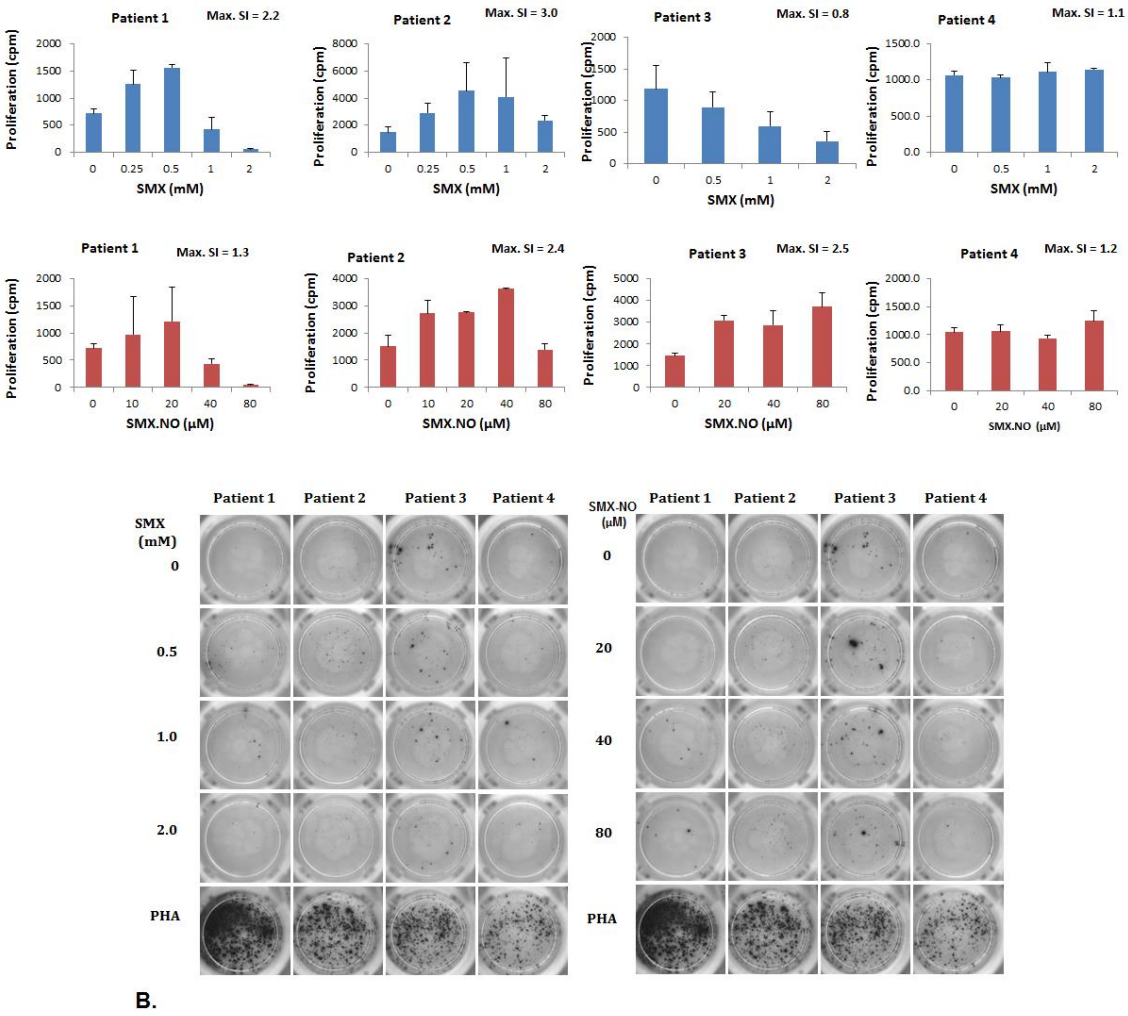


Figure 3.2- LTT and IFN-Ɣ secretion by PBMCs. (A) PBMCs (1.5×10^4 cells, 100 µL) were incubated with graded concentrations of either SMX (0.25-2 mM) or SMX.NO (10-80 µM) in 96-well U-bottom plates. Plates were incubated 37°C under an atmosphere of 95% O_2/5% CO_2 for 5 days. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting. **(B)** The ELISpot plate was pre-coated with human IFN-Ɣ antibody according to manufacturer's instruction and incubated overnight at 4°C. PBMCs (0.5×10^6) were incubated with either SMX (0.5-2 mM) or SMX.NO (20-80 µM) using culture medium as negative control. Plates were then incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. The ELISpot plate was developed according to manufacturer's instruction. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader.

3.4.2 T- Lymphocyte proliferation, cross reactivity and cytokine secretion profile

The antigen specificity of TCCs was determined following serial dilution using proliferation and cytokine secretion as readouts. Clones were generated from PBMCs isolated from four SMX-hypersensitive patients. The average proliferative responses are shown in figure 3.3. A total of 944 clones were tested. Thirty-nine CD4+ clones were SMX.NO responsive, while only one CD8+ clone was activated with SMX (figure 3.4). No cross reactivity was observed with SMX for SMX.NO-specific-TCCs. Similarly, the single SMX-responsive TCC showed no cross-reactivity with SMX.NO (table 3.2 and figure 3.4). Drug-specific TCCs secreted IFN-ϒ, granzyme-B, IL-5 and IL-13 in response to either SMX (2 mM) or SMX.NO (50 μM) as shown in figure 3.5.

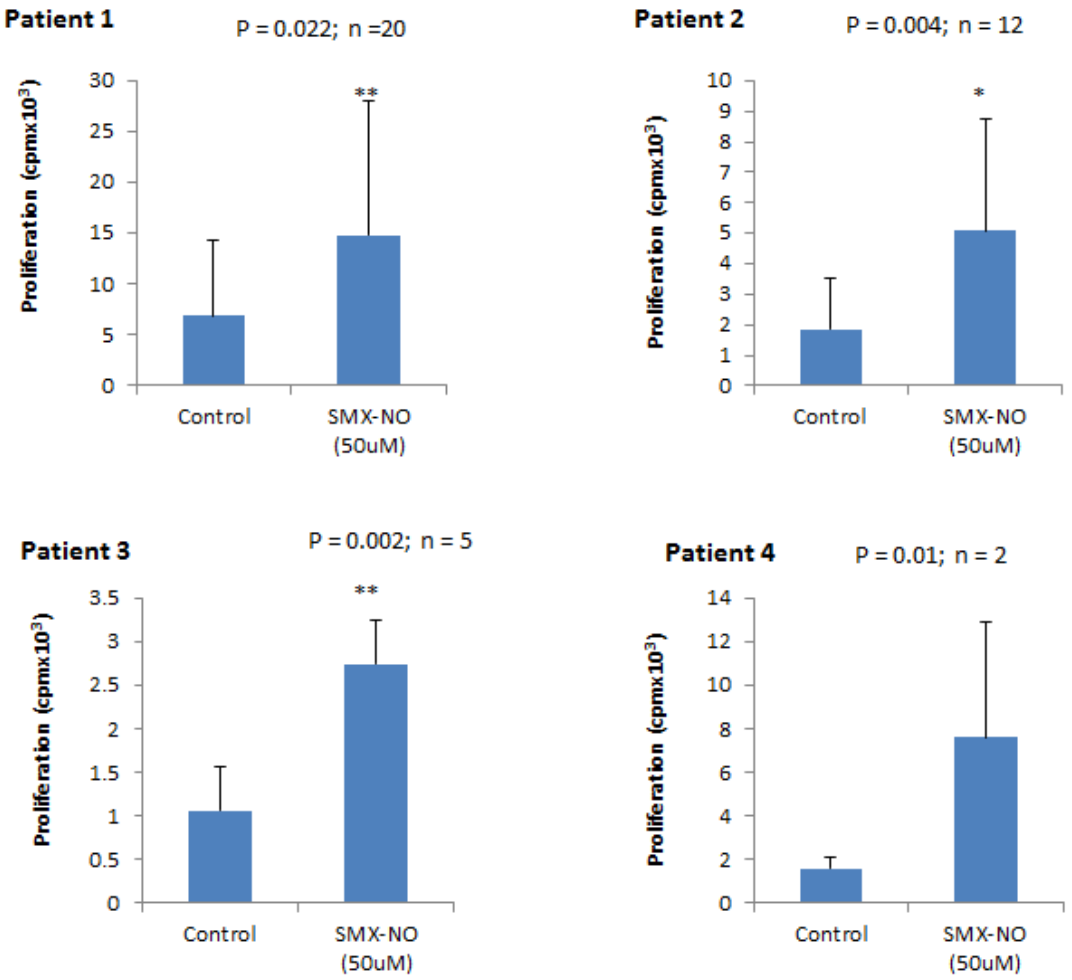


Figure 3.3- T-cell proliferation in response to SMX.NO. T-cell clones (5×10^4 cells, 50 µL) were co-incubated with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells, 50 µL) and SMX.NO (50 µM) in a 96-well U-bottom microplate using T-lymphocyte culture medium as negative control. The plates were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting.

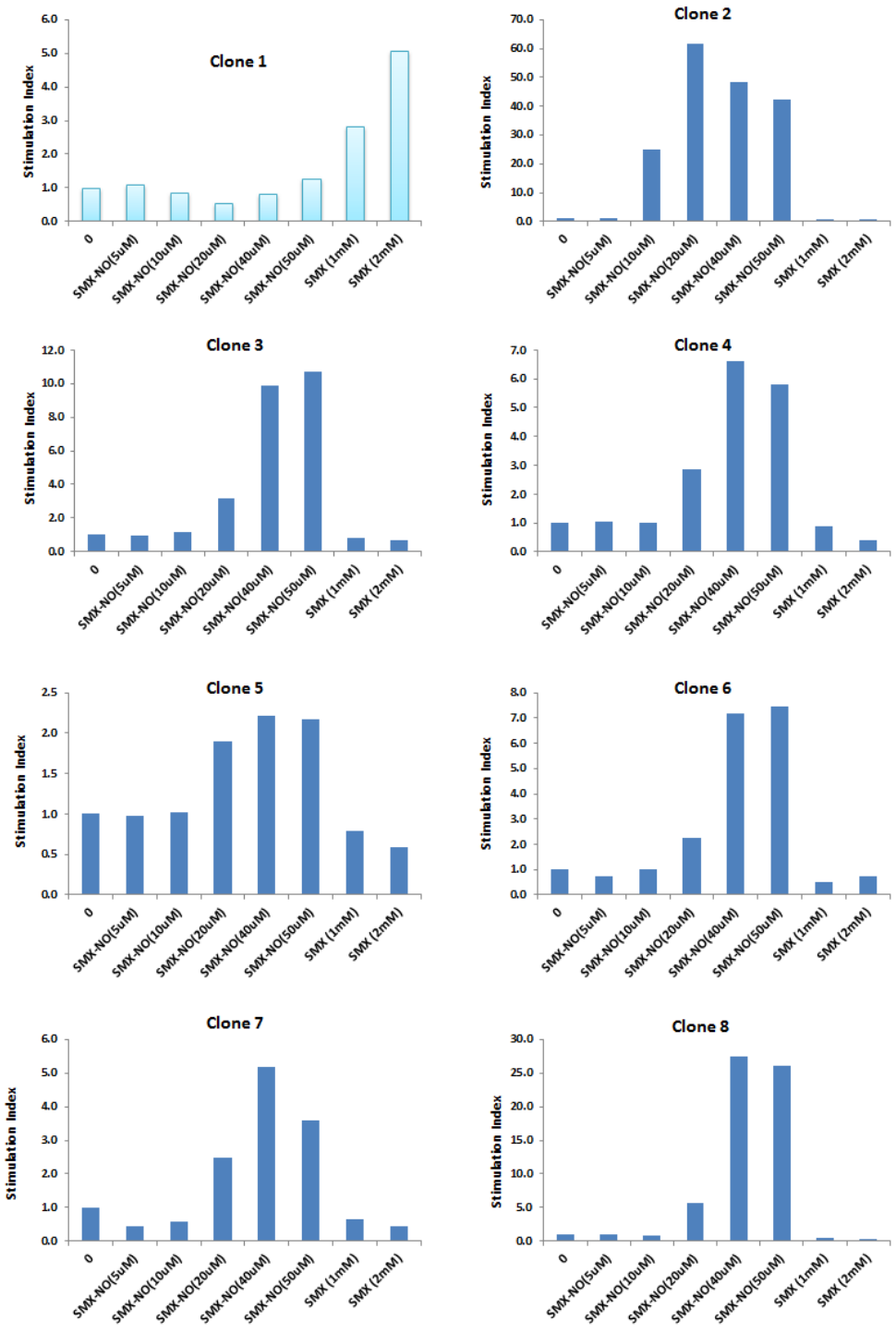


Figure 3.4- T-cell proliferation and cross reactivity. T-cell clones (5×10^4 cells, 50 µL) were co-incubated with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells, 50 µL) in the presence of either SMX or SMX.NO in a 96-well U-bottom microplate using T-lymphocyte culture medium as negative control. The plates were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting. Result shows 8 representative drug-specific TCCs.

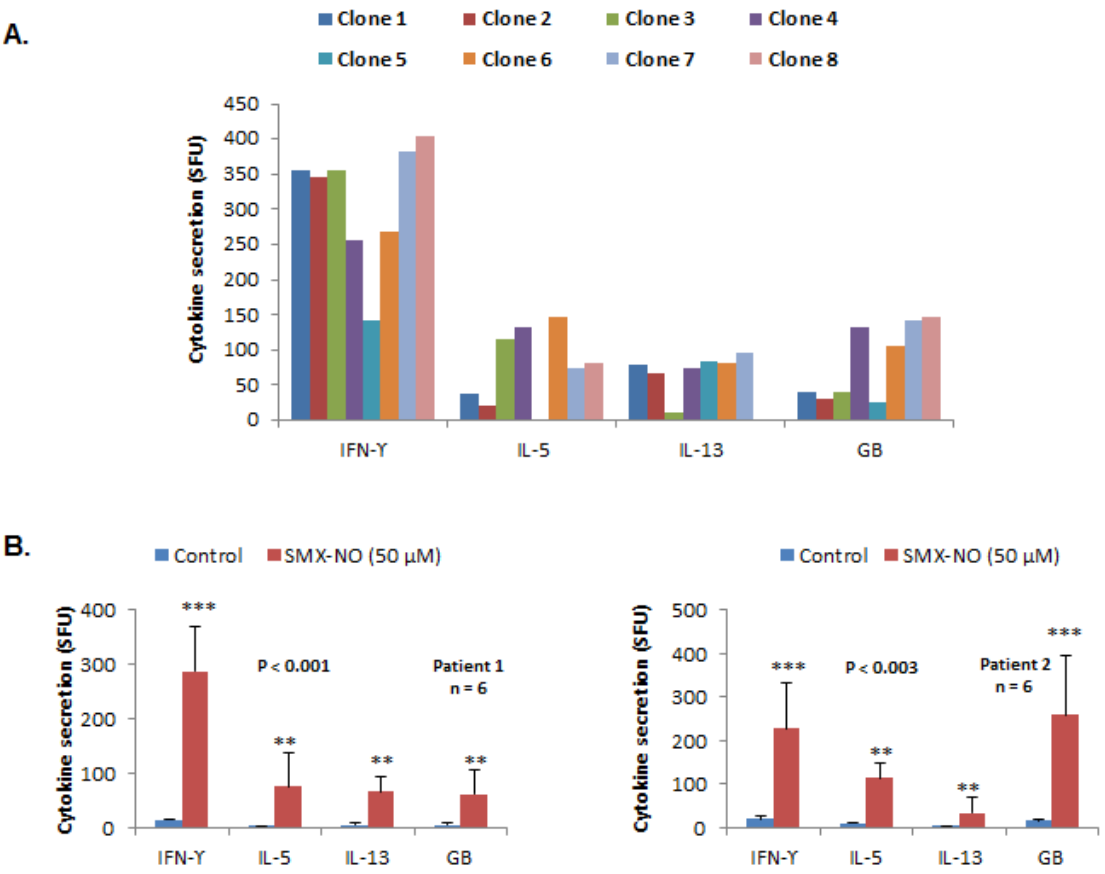


Figure 3.5-Cytokine secretion profiles. (A) Cytokine secretion by eight representative drug-specific TCCs. ELISpot plates were pre-coated with human IFN-Y, IL-5, IL-13 and granzyme-B antibodies according to manufacturer's instruction and incubated overnight at 4°C. T-cell clones (5×10^4, 50 µL) were co-incubated with irradiated autologous EBV-transformed B-cells (1×10^4, 50 µL) and SMX.NO (50 µM) using culture medium as negative control. The plates were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. The ELISpot plates were developed according to manufacturer's instruction. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader. **(B)** Variations in cytokine secretion profile of SMX.NO-specific TCCs generated from Patient 1 and Patient 2.

3.4.3 T-cell CD phenotyping

All the SMX.NO responsive TCCs generated from Patient 1 expressed CD4 cell surface protein. However, the one SMX-responsive TCCs was $CD8^+$. All the clones generated from Patient 2 were SMX.NO responsive and expressed the CD4 cell surface protein (figure 3.6).

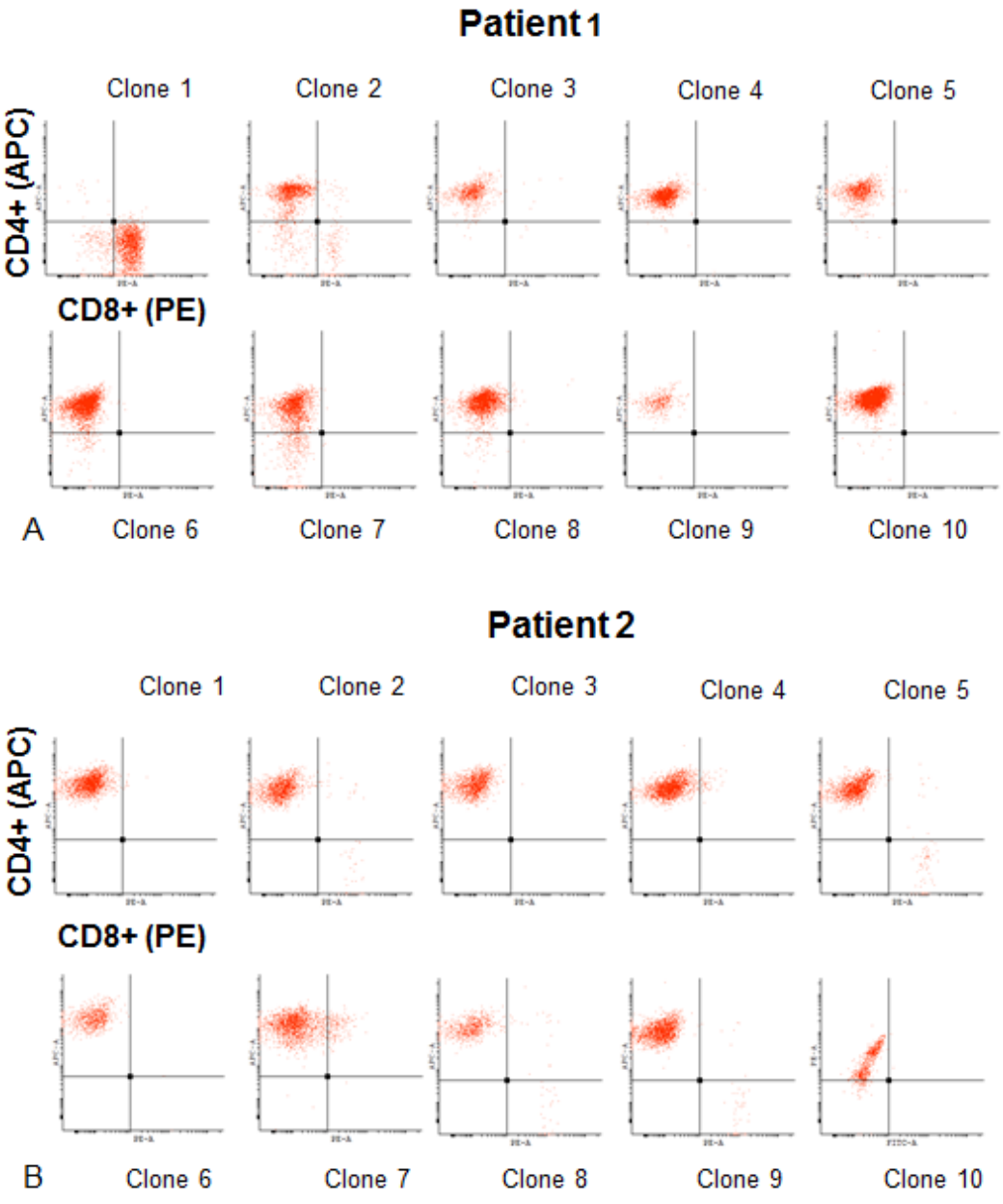


Figure 3.6 T-cell phenotyping for CD4 and CD8 cell surface expression on drug-specific TCCs generated from two SMX-hypersensitive patients. TCC suspension (50 µL) was incubated with both CD4-APC and CD8-PE antibodies for 20 minutes at 4°C. Cells were washed and signals acquired by flow cytometry.

3.4.4 HLA-genotyping

RNA (200 ng/ml) extracted from PBMCs of SMX-hypersensitive patients and SMX-naïve volunteers were genotyped by Histogenetics (USA) at five loci. The choice of the loci to be typed was based on the frequency of their reported involvement in drug hypersensitivity reactions. Three MHC I and two MHC II loci were typed for every patient and volunteer sample (table 3.3).

Table 3.3- HLA genotype for six SMX- hypersensitive patients (01, 02, 03, 05, 06, and 07) and eight SMX-naïve volunteers (04, 08, 09, 10, 11, 12, 13, and 14). SMX.NO-specific TCCs used for HLA-restriction studies were generated from patient 01 and patient 02.

Subject ID	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1
01	A*01:01:01G/A*03:01:01G	B*35:01:01G/B*40:02:01G	C*02:02:02G/C*04:01:01G	DRB1*01:01:01/DRB1*13:01:01	DQB1*05:01:01G/DQB1*06:03:01G
02	A*03:02:01G/A*29:02:01G	B*13:02:01G/B*44:03:01G	C*06:02:01G/C*16:01:01	DRB1*07:01:01G/DRB1*07:01:01G	DQB1*02:01:01G/DQB1*02:01:01G
03	A*23:01:01G/A*30:02:01G	B*18:01:01G/B*44:03:01G	C*04:01:01G/C*05:01:01G	DRB1*03:01:01G/DRB1*07:01:01G	DQB1*02:01:01G/DQB1*02:01:01G
04	A*02:03:01G/A*26:01:01G	B*15:12:01G/B*51:01:01G	C*04:03/C*14:02:01G	DRB1*15:02:01/DRB1*09:01:02	DQB1*03:03:02G/DQB1*05:02:01G
05	A*02:01:01G/A*29:02:01G	B*15:01:01G/B*18:01:01G	C*03:03:01G/C*12:03:01G	DRB1*11:13:01G/DRB1*04:01:01	DQB1*03:01:01G/DQB1*05:03:01G
06	A*01:01:01G/A*01:01:01G	B*08:01:01G/B*15:01:01G	C*04:01:30/C*07:01:12 C*04:01:01G/C*07:01:01G	DRB1*03:01:01G/DRB1*03:01:01G	DQB1*02:01:01G/DQB1*02:01:01G
07	A*02:01:01G/A*24:02:01G	B*18:01:01G/B*40:01:01G	C*03:04:01G/C*05:01:01G	DRB1*15:01:01G/DRB1*13:02:01	DQB1*06:02:01/DQB1*06:04:01G
08	A*02:01G/A*11:01G	B*35:01G/B*44:03G	C*04:01G/C*04:01G	DRB1*01:01:01/DRB1*13:01:01	DQB1*05:01:01/DQB1*06:03:01
09	A*01:01G/A*03:01G	B*18:01G/B*27:05G	C*02:02:02/C*07:01G	DRB1*01:01:01/DRB1*01:01:01	DQB1*05:01:01/DQB1*05:01:01
10	A*01:01G/A*26:01G	B*40:01G/B*57:01:01	C*03:04/C*06:02	DRB1*01:01:01/DRB1*13:01:01	DQB1*05:01:01/DQB1*06:03:01
11	A*01:01:01G/A*02:01:01G	B*44:02:01G/B*57:01:01	C*05:01:01G/C*06:02:01G	DRB1*01:01:01/DRB1*07:01:01G	DQB1*03:03:02/DQB1*05:01:01
12	A*02:01:01G/A*03:01:01G	B*07:02:01G/B*57:01:01	C*06:02:01G/C*07:02:01G	DRB1*15:01:01G/DRB1*07:01:01G	DQB1*03:03:02/DQB1*06:02:01
13	A*02:01G/A*32:01	B*14:01/B*15:01G	C*01:02G/C*08:02	DRB1*03:01:01/DRB1*07:01:01	DQB1*02:01G/DQB1*02:01G
14	A*01:01G/A*01:01G	B*08:01G/B*14:02:01	C*07:01G/C*08:02	DRB1*03:01:01/DRB1*03:01:01	DQB1*02:01G/DQB1*02:01G

3.4.5 MHC-restricted SMX-NO recognition

Anti-human MHC I and MHC II blocking antibodies were used to determine MHC-restricted T-lymphocyte activation in proliferation and ELISpot assays. Proliferation of TCCs was significantly decreased in the presence of an MHC II blocking antibody ($P < 0.0001$; figure 3.7). Furthermore, MHC II blockade resulted in an appreciable decrease in the secretion of both Th1 and Th2 cytokines alongside the cytotoxic molecule; granzyme-B (figures 3.8 and 3.9). In contrast, MHC class I blockade had very little effect. Since most of the clones generated were $CD4^+$ and MHC II restricted, we further investigated the involvement of the different sub-classes of MHC II in SMX.NO recognition by drug-specific T-lymphocytes. Autologous EBV-transformed B-cells were pre-incubated with anti-HLA-DP, DQ or DR antibodies for 30 minutes and then co-cultured with SMX.NO responsive TCCs. The majority (86%) of the SMX.NO-specific TCCs were HLA-DQ restricted ($P \leq 0.005$) while 14% showed HLA-DR restriction. HLA-DQ blockade resulted in a significant decrease in T-lymphocyte proliferation and a modest decrease in IFN-ϒ, IL-5, IL-13 and granzyme-B in most of the TCCs (figures 3.10 and 3.11).

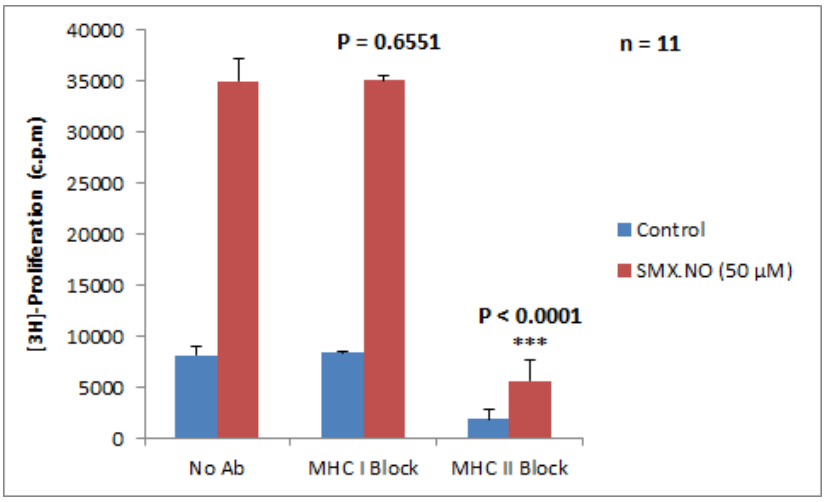


Figure 3.7-MHC blocking proliferation assay. Autologous EBV-transformed B-cells (1×10^4 cells) were pre-incubated with either MHC I or MHC II blocking antibodies (5 µg/mL) for 30 minutes at 37°C under an atmosphere of 95% O_2/5% CO_2, then co-cultured with SMX.NO-specific TCCs in the presence or absence of SMX.NO (50 µM) in a 96-well microplate. The plate was then incubated for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added to each well for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting.

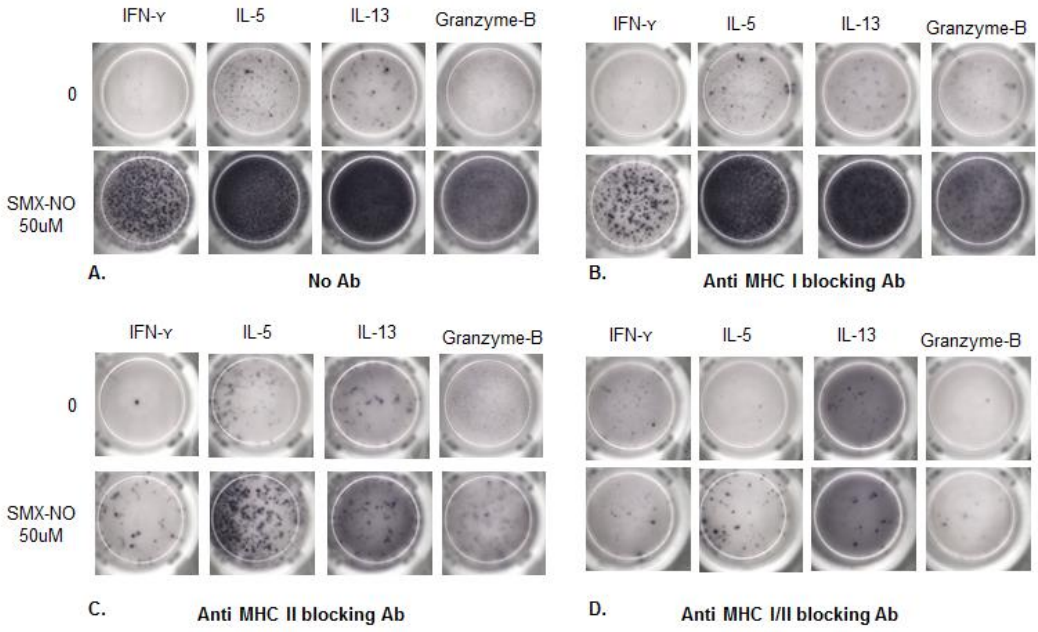


Figure 3.8-MHC blocking cytokine assay. Cytokine secretion profile of representative T-cell clone in the presence of medium control, MHC I, MHC II or a combination of both MHC I and MHC II antibodies (5 µg/mL), (A-D). ELISpot plates were coated with human IFN-Y, IL-5, IL-13 and Granzyme B coating antibodies and incubated at 4°C overnight. Irradiated autologous EBV-transformed B-cells (1×10^4, 50 µL) were pre-incubated with either MHC I, MHC II, or both blocking antibodies for 30 minutes at 37°C under an atmosphere of 95% O_2/5% CO_2, then co-cultured with SMX.NO-specific T-cell clones (5×10^4, 50 µL) in the presence or absence of SMX.NO (50 µM) in a U-bottom 96-well plate. The plates were incubated at 37°C, 5% CO_2 under an atmosphere of 95% O_2/5% CO_2 for 48 hours. Plates were developed according to manufacturer's instructions and spots visualised using an AID ELISpot reader. Data is representative of three SMX-NO-specific TCCs tested.

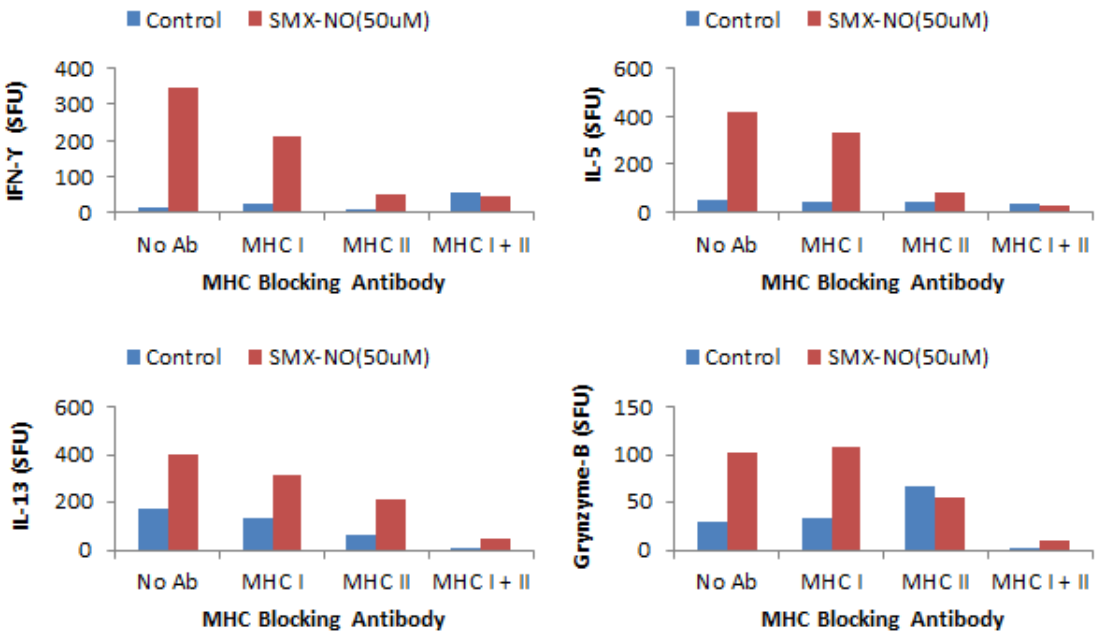


Figure 3.9-Graphs showing spot counts (cytokine secreting cells) representing various conditions of treatment presented in figure 3.8.

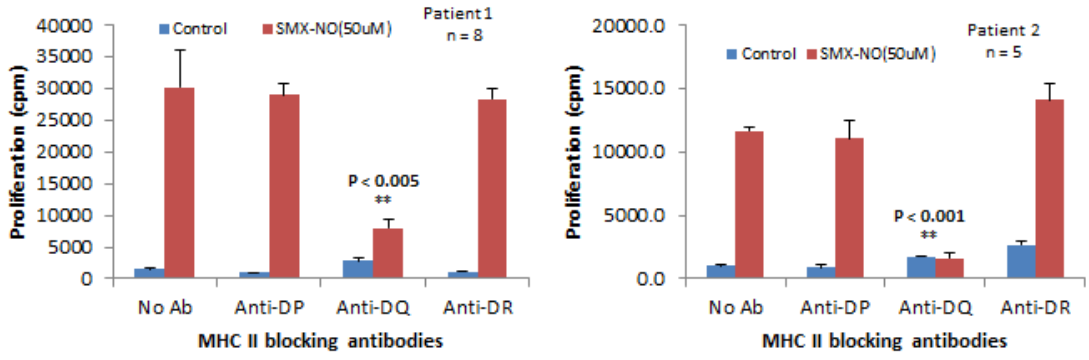


Figure 3.10-HLA-DP, -DQ and –DR restricted SMX.NO presentation to TCCs. Irradiated autologous EBV-transformed B-cells (1×10^4 cells, 50 µL) were pre-incubated with anti-HLA-DP, -DQ or –DR blocking antibodies (5 µg/mL) for 30 minutes. APCs were then co-incubated with drug-specific TCCs (5×10^4 cells, 50 µL) in the presence or absence of SMX.NO (50 µM) in a 96-well U-bottom microplate using culture medium as negative control. The plate was incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0. 5µCi) was added to each well for the final 16 hours of incubation and T-lymphocyte proliferation evaluated using scintillation counting. Bar chart illustrates data from 8 and 5 SMX.NO-specific TCCs from patients 1 and 2 respectively.

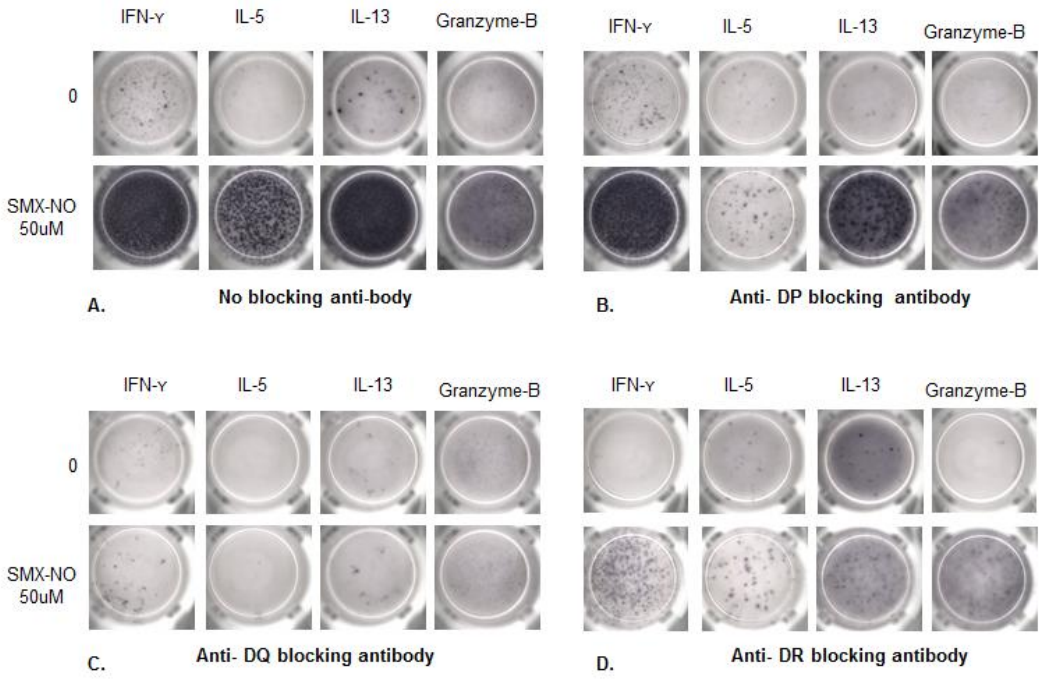


Figure 3.11- HLA-DP, -DQ and –DR restricted SMX.NO presentation to TCCs. ELISpot plates were coated with human IFN-γ, IL-5, IL-13 and Granzyme-B coating antibodies overnight at 4°C. Irradiated autologous EBV-transformed B-cells (1×10^4 cells) were pre-incubated with culture medium (control), anti-HLA-DP, -DQ or –DR blocking antibodies (5 µg/mL), A-D, for 30 minutes at 37°C under an atmosphere of 95% O_2/5% CO_2 and then co-cultured with SMX.NO-specific TCCs (1×10^4) in the presence or absence of SMX.NO (50 µM) using a U-bottom 96-well plate. Plates were then incubated for 48 hours. Plates were then developed according to manufacturer's instructions and spots visualised using an AID ELISpot reader.

3.4.6 APC mismatch assay

APC mismatch experiments involved incubation of SMX.NO-specific TCCs with either autologous or heterologous APC to verify the role of specific HLA alleles in T-cell activation. Fast growing SMX.NO-specific TCCs generated from Patient 1 and Patient 2 were used for the APC mismatch assay. Heterologous APCs utilised for this experiment were generated from PBMCs of SMX-naïve volunteers (see table 3.3). All APCs used were EBV-transformed B-cells. TCCs were assessed based on their proliferative capacity and cytokine secretion profile in the presence of either autologous or various heterologous APCs (figure 3.12 - 3.15). SMX.NO-specific TCCs from either Patient 1 or Patient 2 proliferated when SMX.NO was presented on volunteer or patient APC

expressing similar HLA-DQB1 genotype (HLA-DRB1*05:01:01 for Patient 1 and HLA-DQB1*02:01:01 for patient 2) as the autologous EBV-transformed B-cells from the patient in question (table 3.4 and 3.5). A similar trend was observed with cytokine secretion profile in the presence of APCs expressing similar HLA-DQB1 genotype as the patient. The choice of HLA-DQB1 gene was based on the HLA- DQ restricted recognition of SMX.NO observed in the majority of SMX.NO-specific TCCs (figures 3.10 and 3.11). Table 3.4 and 3.5 summarise the similarities in the HLA-DQB1 gene (red highlights) expressed by autologous APCs (01 and 02) and heterologous APCs (others). Interestingly, Patient 1 and all volunteers expressing the HLA-DQB1*05:01:01 allele also expressed HLA-DRB1*01:01:01 while Patient 2 and 50% volunteers expressing the HLA-DQB1*02:01:01 allele also expressed HLA- DRB1*07:01:01 allele.

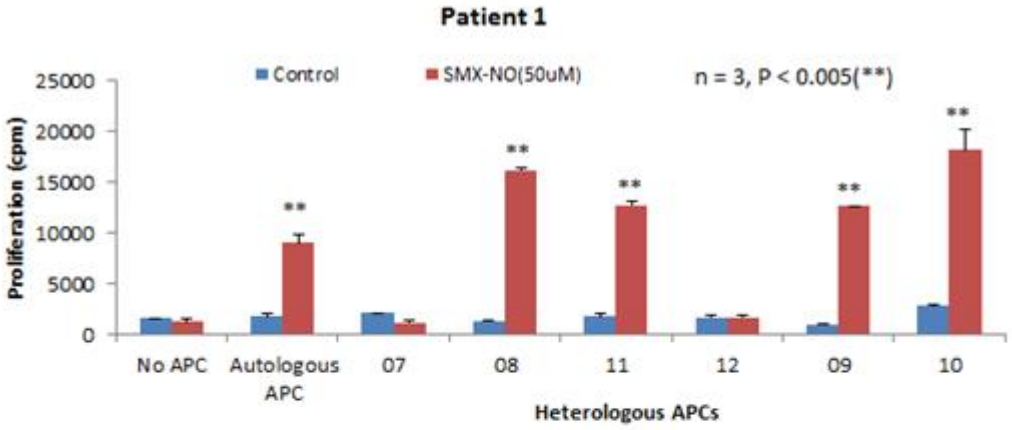


Table 3.4-HLA genotype for Patient 1 and SMX-naïve volunteers

Subject ID	HLA-DQB1
01 (Autologous APC)	DQB1*05:01:01G/DQB1*06:03:01G
07	DQB1*06:02:01/DQB1*06:04:01G
08	DQB1*05:01:01/DQB1*06:03:01
09	DQB1*05:01:01/DQB1*05:01:01
10	DQB1*05:01:01/DQB1*06:03:01
11	DQB1*03:03:02/DQB1*05:01:01
12	DQB1*03:03:02/DQB1*06:02:01

Figure 3.12-APC mismatch proliferation assay of a representative TCC from Patient 1. Irradiated autologous or heterologous EBV-transformed B-cells (1×10^4 cells, 50 µL) were co-cultured with drug-specific TCCs (5×10^4 cells, 50 µL) in the presence or absence of SMX.NO (50 µM) in a 96-well U-bottom microplate using culture medium as negative control. The plate was incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added to each well for the final 16 hours of incubation and T-lymphocyte proliferation evaluated using scintillation counting. Table 3.4 (above) shows HLA genotype from naïve volunteers

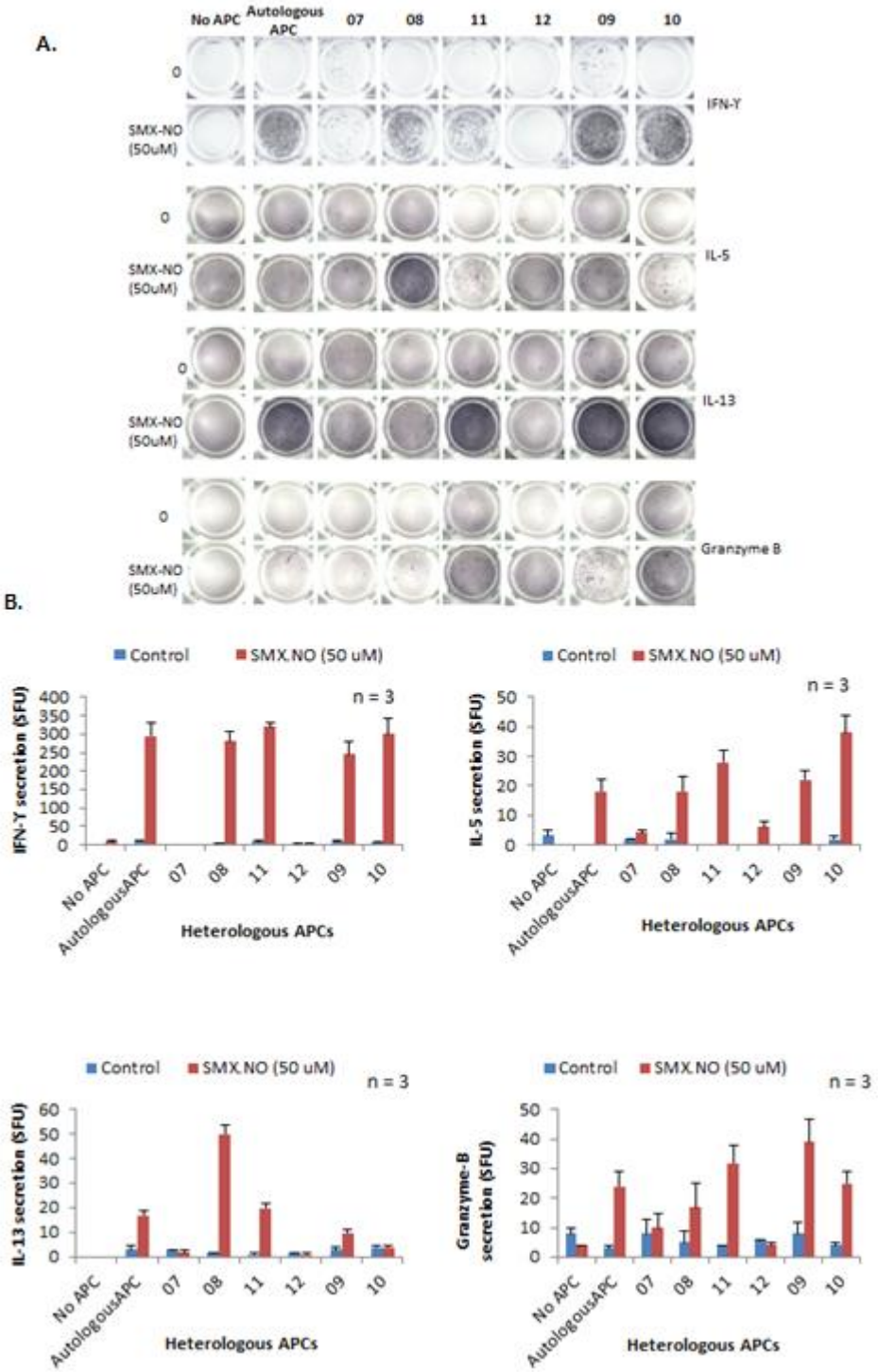


Figure 3.13-APC mismatch ELISpot assay of a representative SMX.NO-specific TCC from Patient 1. (A) ELISpot plates were coated with human IFN-Y, IL-5, IL-13 and Granzyme-B coating antibodies overnight at 4°C. Irradiated autologous or heterologous EBV-transformed B-cells (1×10^4 cells, 50 µL) were co-cultured with SMX.NO-specific T-cell clones (5×10^4 cells, 50 µL) with or without SMX.NO (50 µM, 100 µL) using a U-bottom 96-well microplate. The plate was incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. ELISpot plate was then developed according to manufacturer's instructions and spots visualised using an AID ELISpot reader. **(B)** Bar charts representing spot counts in ELISpot images shown above.

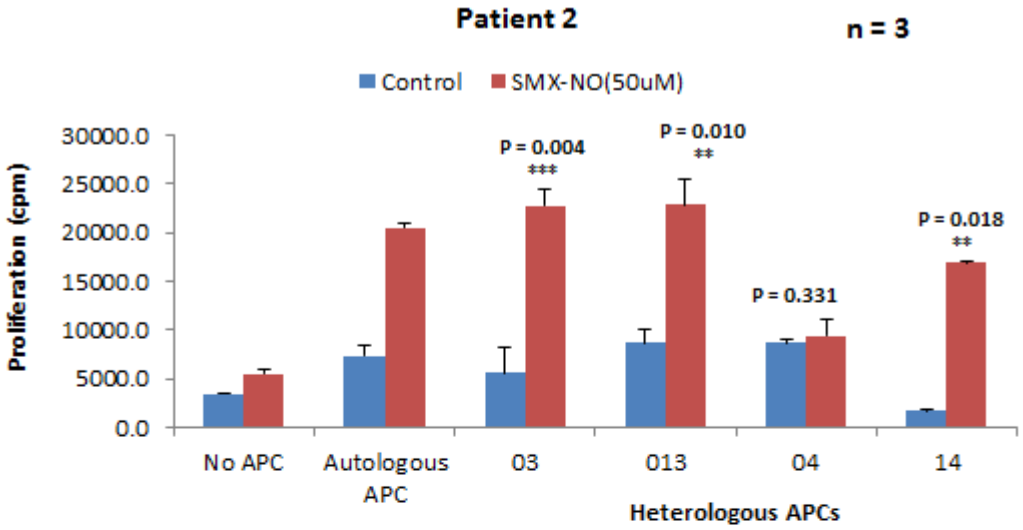


Table 3.5- HLA-genotype for Patient 2 and SMX-Naïve volunteers

Subject ID	HLA-DQB1
02 (Autologous APC)	DQB1*02:01:01G/DQB1*02:01:01G
03	DQB1*02:01:01G/DQB1*02:01:01G
04	DQB1*03:03:02G/DQB1*05:02:01G
13	DQB1*02:01G/DQB1*02:01G
14	DQB1*02:01G/DQB1*02:01G

Figure 3.14-APC mismatch proliferation assay of a representative SMX.NO specific TCC from Patient 2. Irradiated autologous or heterologous EBV-transformed B-cells (1×10^4 cells, 50 µL) were co-cultured with drug-specific T-cell clones (5×10^4 cells, 50 µL) in the presence or absence of SMX.NO (50 µM) in a 96-well U-bottom microplate using culture medium as negative control. The plate was incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added to each well for the final 16 hours of incubation and T-lymphocyte proliferation evaluated using scintillation counting.

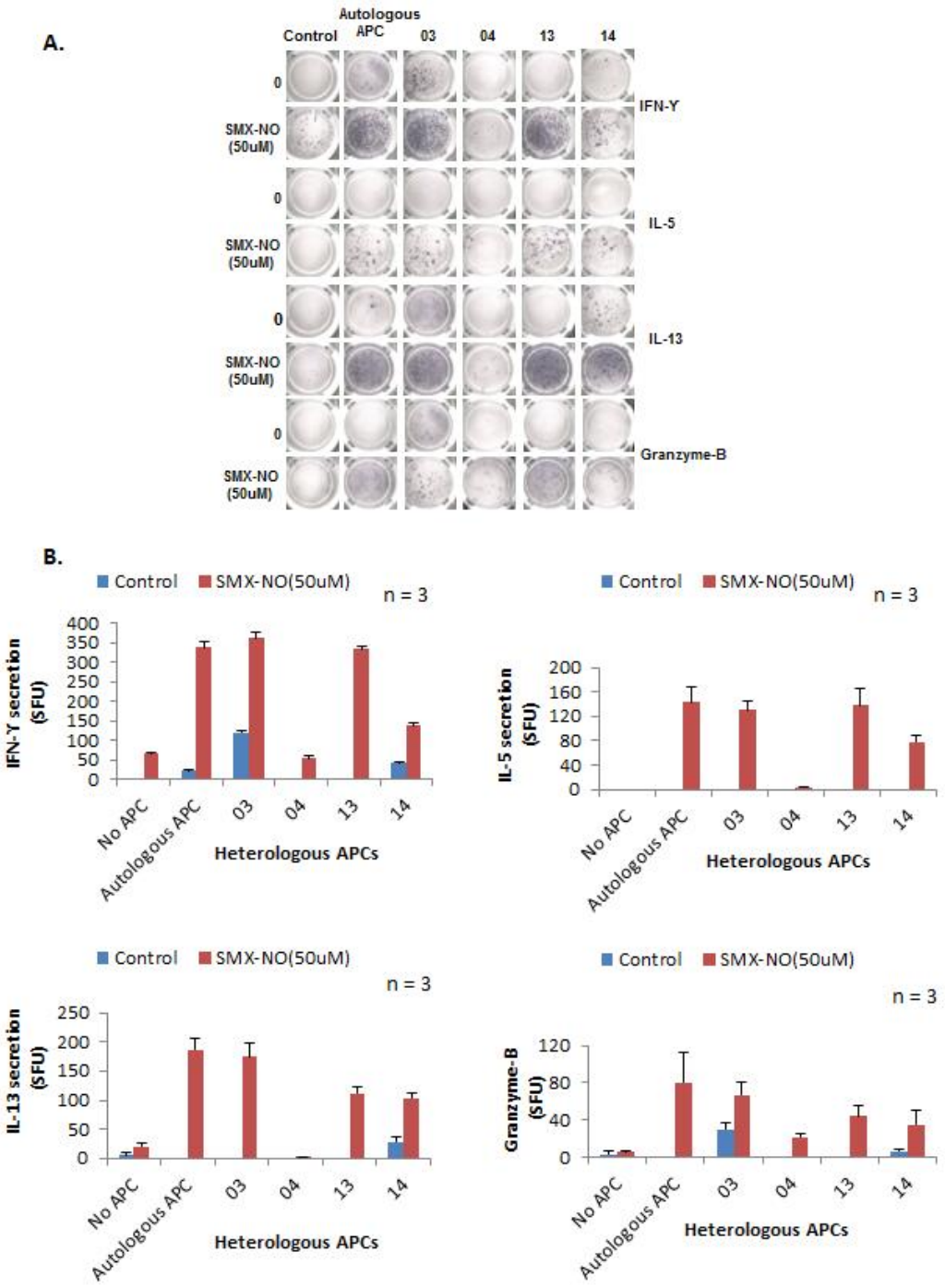


Figure 3.15-APC mismatch ELISpot assay of a representative SMX.NO-specific TCC from Patient 2. (A) ELISpot plates were coated with human IFN-Y, IL-5, IL-13 and Granzyme-B coating antibodies overnight at 4°C. Irradiated autologous or heterologous EBV-transformed B-cell line (1×10^4 cells, 50 µL) were co-cultured with SMX.NO-specific T-cell clones (5×10^4 cells, 50 µL) with or without SMX.NO (50 µM, 100 µL) using a U-bottom 96-well microplate. The plate was incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. ELISpot plate was then developed according to manufacturer's instructions and spots visualised using an AID ELISpot reader. **(B)** Bar charts representing spot counts in ELISpot images shown above.

3.4.7 Self-presentation of antigen by SMX.NO responsive T-cell clone

Forty-nine SMX.NO specific TCCs were generated. Thymidine proliferation and IFN-ϒ data revealed that one of the TCCs was stimulated with SMX.NO in the absence of APCs (figure 3.16A and B). Evaluation of MHC I and MHC II expression on TCCs revealed uniform expressions of both MHC I and MHC II protein on all of the three SMX.NO-specific TCCs examined (one self-presenting TCC and two non-self-presenting TCCs; figure 3.16 C and D). MHC II expressed on Clone 1 is most likely responsible for the activation with SMX.NO in the absence of APC; however, it is theoretically possible that SMX.NO stimulates TCCs directly through the TCR. All three clones analysed were $CD4^+$ TCCs (figure 3.6).

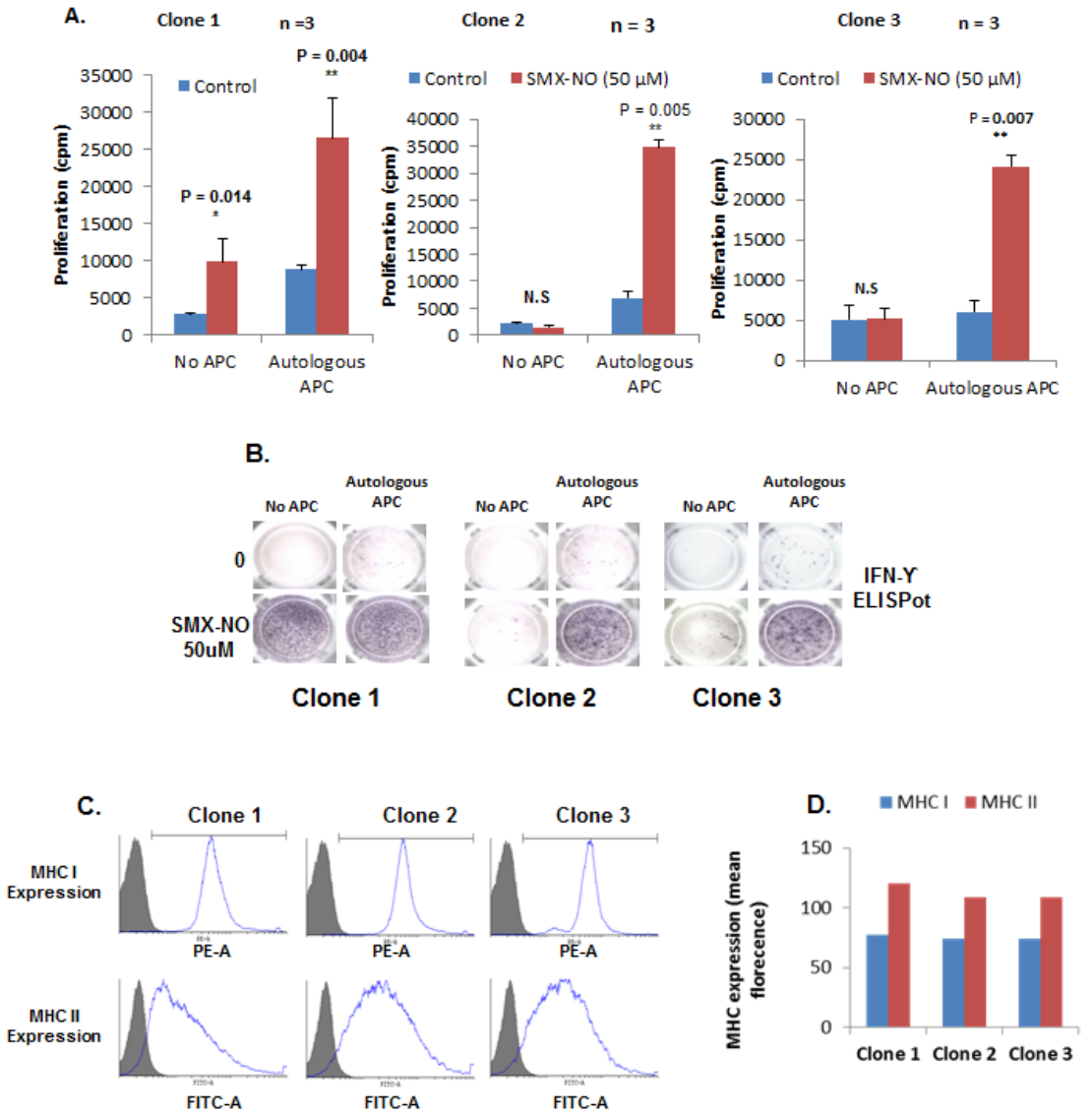


Figure 3.16 Activation of SMX.NO responsive TCC in the absence of APC. (A) Thymidine proliferation assay for three SMX.NO-specific $CD4^+$ TCCs. SMX.NO-specific T-cell clones (5×10^4 cells, 50 µL) were co-cultured with or without irradiated autologous EBV-transformed B-cells (1×10^4 cells, 50 µL) in the presence or absence of SMX.NO (50 µM, 100 µL) in a 96-well U-bottom microplate using culture medium as negative control. The plate was incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting. **(B)**. Interferon gamma ELISpot. SMX.NO-specific T-cell clones (5×10^4) and EBV-transformed B-cells (1×10^4) were co-cultured in a similar manner as stated in the proliferation assay above in an ELISpot plate pre-coated with IFN-gamma capture antibody. The plate was incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours and then developed according to manufacturer's instructions and spots visualised using an AID ELISpot reader **(C).** MHC I and MHC II phenotyping on three SMX.NO specific TCCs. TCC suspension (50 µL) was incubated with both MHC I-PE and MHC II-FITC antibodies for 20 minutes at 4°C. Cells were washed with FACS buffer (1ml) and resuspended in FACS buffer (200 µL). MHC I and MHC II expressions were investigated by flow cytometry and data analyzed by Cyflogic. Grey shades represent baseline auto-florescence while blue lines represent either MHC I or MHC II expression. **(D).** Bar charts showing relative MHC I and MHC II expression on the three SMX.NO-specific TCCs.

3.4.8 T-cell Vβ receptor analysis

Examination of T-cell receptor expression on sixteen SMX.NO-specific TCCs clones generated from Patient 1 (P1) and Patient 2 (P2) revealed a distribution of TCR expression across five V_β subclasses. TCCs generated from Patient 1 expressed five different V_β repertoires (V_β2, V_β3, V_β7.1, V_β13.1 and V_β14; figure 3.17). In contrast, 100% of clones generated from Patient 2 (P2) expressed the V_β2 TCR. Taken together, the percentage of V_β T-cell receptor usage observed in the 16 SMX.NO-specific TCCs were V_β2 (69%), V_β7.1 (13%), V_β13.1 (6%), V_β3 (6%) and V_β14 (6%) (Figure 3.18 B).

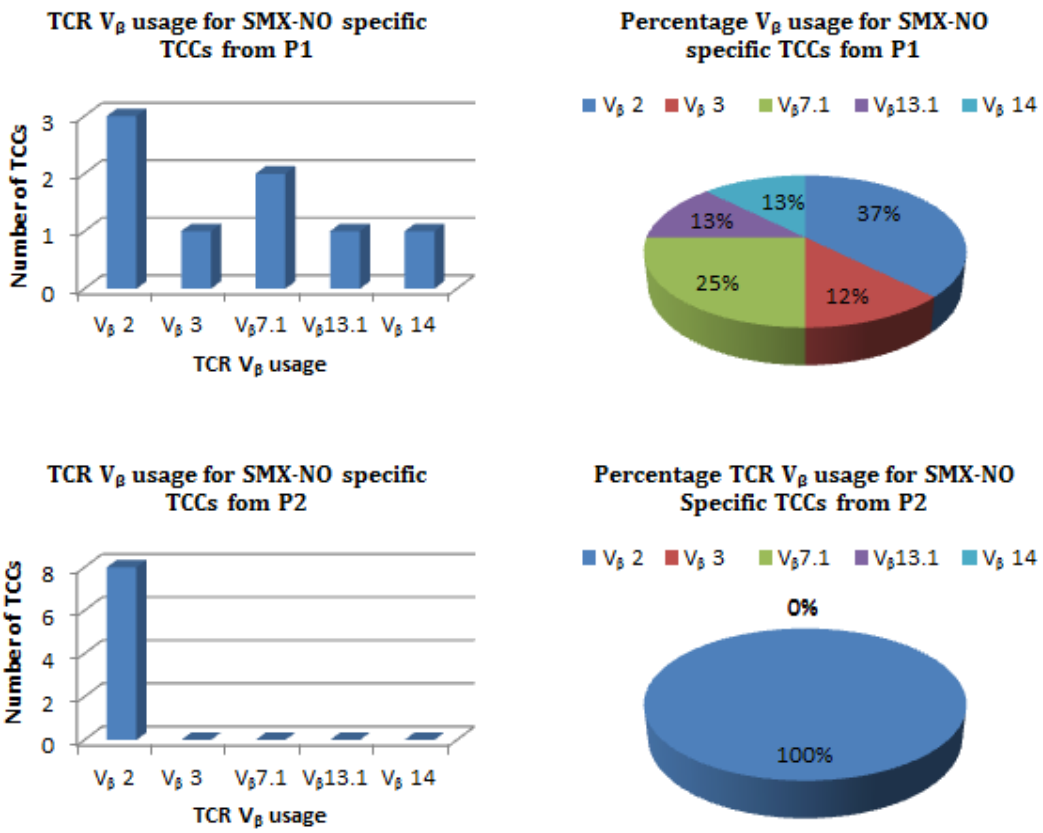


Figure 3.17- TCR V_β analysis. T-cell suspensions (100 µL) were incubated with various TCR Vβ antibodies and TCR Vβ usage determined using flow cytometry and data analyzed by Cyflogic.

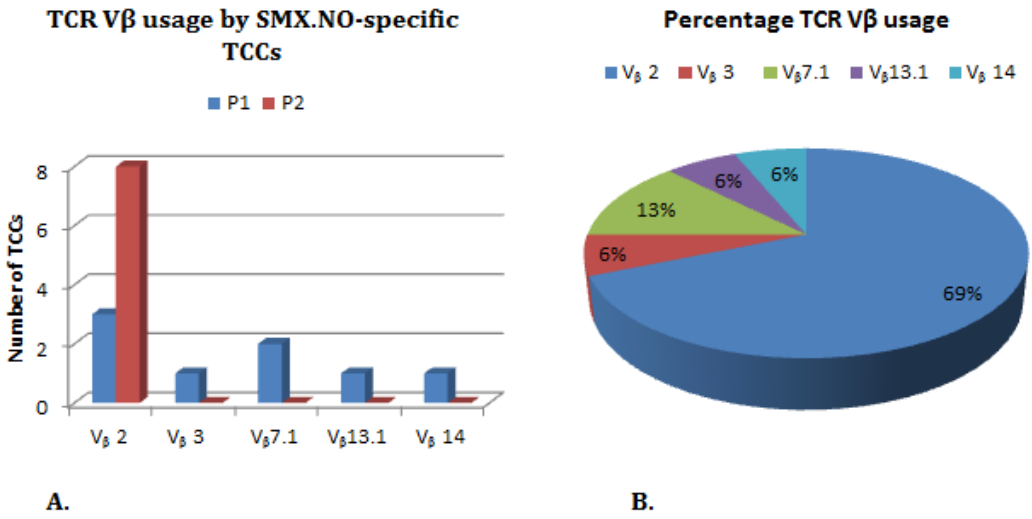


Figure 3.18- (A) Graphical representation of TCR V_β usage of SMX.NO-specific TCCs generated from 2 SMX-hypersensitive patients (P1 and P2). **(B).** Percentage TCR V_β expression of 16 SMX.NO-specific TCCs.

3.5 Discussion

Drug hypersensitivity reactions can be serious, fatal and places an enormous burden on the health care delivery system (Lazarou et al. 1998; Pirmohamed et al. 2004; Davies et al. 2009). A number of studies have focused on the role of the HLA allele in drug-induced hypersensitivity reactions (Chung et al. 2004; Mallal et al. 2008; Daly et al. 2009; Illing et al. 2012; Yun et al. 2012) in order to delineate the complex structural interactions between the drug molecule, the MHC-peptide complex and the T-cell receptor. Off-target drug reactions resulting from many small molecules involve a specific interaction with HLA molecules (covalent or labile) followed by T-cell stimulation. Other risk factors associated with hypersensitivity drug reactions include: the chemical properties of the drug (Guglielmi et al. 2006), viral infections such as HIV and herpes viruses (Coopman et al. 1993; Shiohara et al. 2006), gender (Schmid et al. 2006; Thong and Tan 2011), genetic predisposition other than HLA (Kim et al. 2010) and T-cell receptor repertoire (Ko et al. 2011).

Originally, SMX recognition by TCR was explained mainly by the hapten model; however, Schnyder and his colleagues later described a MHC-restricted but metabolism- and processing-independent pathway of drug-specific T-cell activation (Schnyder et al. 2000). Their findings revealed a non-covalent, low-affinity interaction between SMX and MHC-peptide complexes on APCs. They reported that while the majority of TCCs generated from hypersensitive patients were SMX-specific, a small percentage responded to SMX.NO and were cross-reactive. Although, they concluded that the same TCR can recognise SMX-derived antigens either covalently or non-covalently bound to the MHC-peptide, the molecular mechanisms involved in such dual activations are unclear.

In contrast, 98% of the TCCs generated from four SMX-hypersensitive patients in this study were SMX.NO-specific and showed no cross-reactivity with SMX. This is consistent with the dogma that SMX becomes immunogenic only after oxidative metabolism that generates the protein reactive metabolite, SMX.NO (Park et al. 1998). Only 1/40 of the drug-specific TCCs was SMX-responsive but showed no cross-reactivity with SMX.NO, suggesting a direct activation of SMX-specific TCCs as described above.

The MHC restriction pattern of SMX-responsive TCCs has been reported previously (von Greyerz et al. 2001). Most of the SMX-specific clones displayed MHC allele unrestricted drug recognition. In contrast, the MHC restriction pattern of SMX.NO has not been studied. Therefore, this chapter investigated the role of HLA-DQB1 restriction in SMX.NO recognition by drug-specific TCCs.

The SMX.NO-specific TCCs expressed the CD4 cell surface protein. TCR activation of the TCCs resulted in the secretion of IFN-ϒ, IL-5, IL-13, granzyme-B and Fas ligand. The involvement of cytotoxic $CD4^+$ T-cells in drug-induced cutaneous reactions has been reported elsewhere (Schnyder et al. 1998; Hari et al. 2001). MHC restriction experiments revealed that all the $CD4^+$ TCCs were MHC II restricted. A significant decrease in T-cell proliferation and cytokine secretion was also observed when autologous APCs were pre-incubated with an HLA-DQ blocking antibody before a 48 hour co-culture with SMX.NO-responsive T-cell clones. Based on the HLA-DQ restriction of drug-specific TCCs from patient 1 and patient 2, EBV-transformed B-cells generated from PBMCs isolated from SMX-naïve donors expressing matched/partly matched or

unmatched HLA-DQB1 molecules and used as APCs in [^{3}H]-proliferation and ELISpot cytokine experiments (APC mismatch experiments).

Interestingly, APCs generated from volunteers expressing the same HLA-DQB1 genotype as either patient 1 or patient 2 presented SMX.NO to drug-specific TCCs and triggered significant T-cell proliferation and cytokine secretion comparable to autologous APCs. APCs from volunteers expressing dissimilar HLA-DQB1 genotype failed to present SMX.NO to drug specific T-cell clones. These data strongly suggest an HLA-DQB1*05:01:01 restricted SMX.NO presentation to TCCs generated from patient 1 and HLA-DQB1*02:01:01 for TCCs generated from patient 2. These data imply that restriction would differ depending on the HLA-DQB*1 gene expressed by individual patients.

Remarkably, Patient 1 and all volunteers expressing the HLA-DQB1*05:01:01 allele also expressed HLA-DRB1*01:01:01 while Patient 2 and 50% volunteers expressing the HLA-DQB1*02:01:01 allele also expressed HLA-DRB1*07:01:01 allele. This suggests the involvement of a haplotype in the observed HLA-restricted SMX.NO presentation of SMX.NO to drug-specific T-lymphocytes. Similar findings have been reported elsewhere for HLA-DRB1 and HLA-DQB1 alleles involved in narcolepsy and Type 1 diabetes (Temajo and Howard 2009; Han et al. 2012). Han et al reported that although the deficiency of hypocretin in narcolepsy patients of Japanese, Korean and Caucasian origins was associated with DRB1*15:01-DQA1*01:02-DQB1*06:02 haplotype, it is the expression of DQB1*06:02 not DRB1*15:01 that is associated with narcolepsy (Han et al. 2012). Hence, in the absence of HLA-DQB1*06:02, individuals that expressed HLA-DRB1*15:01 were not susceptible to narcolepsy.

The vast majority of SMX.NO-responsive TCCs were activated only in the presence of professional APCs. However, one clone was activated with SMX.NO in both the presence and absence of APCs. The clinical and mechanistic implications of such 'self-presentation' have not been clearly defined and remain a subject of speculation. FACS analysis revealed a uniform expression of MHC I and MHC II molecules regardless of whether the TCC 'self-presented' the antigen or not. Hence, the mechanism of T-cell activation through 'self-presentation' may involve an irreversible binding of SMX.NO to embedded peptides in TCC MHC II or a direct TCR modification.

Put together, the APC mismatch data suggested an HLA-restricted SMX.NO presentation, thus contrasting data presented elsewhere that suggests no strict HLA restriction for SMX recognition by T-cells was found (Zanni et al. 1999; von Greyerz et al. 2001). The differences reported may be due to the molecular basis of antigen presentation to SMX- and SMX metabolite-specific T-cells and also differences in the TCR repertoire expressed by TCCs utilised for such HLA-restriction experiments. The role of specific TCR repertoires in certain immune-mediated drug reactions remains unclear due to the large number of T-cell receptor repertoires that exists (Ko et al. 2011). Experiments involving transfection of selected TCRs into hybridoma cells that do not express human MHCs has underscored the involvement of specific TCRs in the interaction with drug molecules (Depta et al. 2004; Schmid et al. 2006). The authors concluded that TCRs are a major determinant of T-cell reactivity to SMX and other structurally related drugs.

Thus, we also investigated the distribution of TCR repertoire on 16 SMX.NO-responsive TCCs generated from 2 subjects. SMX.NO-specific TCCs expressed 5 different TCRV_β repertoires (V_β2, V_β3, V_β7.1, V_β13.1 and V_β14) with majority of the clones analysed expressing V_β2 (69%). T-cell clones expressing V_β2 have been extensively characterised. They are implicated in a number of immune related diseases and cutaneous allergic reactions (Reantragoon et al. 2012; Watkins and Pichler 2013). *In silico* docking studies suggests that the CDR2 and CDR3 regions of SMX-responsive clones expressing TCRV_β2 are critical for SMX interaction (Watkins and Pichler 2013). Importantly, if we assume that the nitroso group of SMX.NO binds irreversibly to the cysteine residue of HLA-DQ binding peptides. Therefore, the pharmacophore that interacts with the TCR will be very similar to the parent compound. Hence, it is possible that SMX.NO modified HLA-restricted peptides dock with TCR in a similar fashion to that described with SMX. Collectively, the restricted but variable distribution of TCRV_β repertoire among SMX.NO-specific TCCs suggests that the HLA molecule and TCR repertoire are important determinants in the pathogenesis of drug-induced hypersensitivity reactions. Further research into delineating the molecular mechanisms of HLA-associated drug-induced hypersensitivity reactions will be important in the design of safer therapeutic agents.

Chapter 4: Activation of naïve and memory T-cells by sulphamethoxazole and nitroso-sulphamethoxazole

Contents

4.1 Introduction

ADRs are a major concern when drugs are used for the prevention and treatment of diseases. Although most ADRs are an extension of the normal pharmacology of the 'culprit drug', a minority of them are idiosyncratic. These reactions are difficult to predict during the research and development of new chemical entities/drugs. This is made even more difficult because some of these reactions are linked with particular HLAs, sometimes expressed by specific populations (Lonjou et al. 2008; Kim et al. 2010; McCormack et al. 2011; Han et al. 2012). The role of specific HLAs in drug hypersensitivity reactions cannot be overemphasised and has been discussed extensively in chapter 3.

T-cells are a critical component in the clinical presentation of hypersensitivity reactions (Schnyder et al. 1998; Naisbitt et al. 2007; Hausmann et al. 2010; Adam et al. 2011). The specificity of an immune-mediated drug reaction is dependent on two major components, namely: drug molecule and the peptides displayed by specific HLA molecules. T-cells recognise drugs that bind to the peptide-MHC complex. This binding may involve the formation of an irreversible covalent bond between the drug molecule and the MHC or embedded peptides (Landsteiner and Jacobs 1935; Weltzien et al. 1996).

Chemically inert drugs also bind to MHC peptide complexes in a reversible, non-covalent manner (Engler et al. 2004). SMX is chemically inert and only acquires protein reactivity after enzyme-induced biotransformation (Cribb and Spielberg 1992; Naisbitt et al. 1999). The processing and the presentation of SMX-protein adduct and drug-specific T-cell activation has been discussed extensively in chapter 1. T-cells that are responsive to SMX and SMX.NO have

been isolated from blood and skin of hypersensitive patients (Schnyder et al. 2000; Naisbitt et al. 2002; Nassif et al. 2002). The initial analysis of 222 drug-specific TCCs generated from various SMX-hypersensitive patients revealed that approximately 97% were SMX responsive and showed no cross-reactivity with SMX.NO (Schnyder et al. 2000). In a similar study by Castrejon et al (2004), drug specific TCCs generated from 3 SMX-hypersensitive patients were evaluated for antigen specificity. They generated and tested a total of 480 TCCs and reported that 44% of the TCCs responded to SMX.NO, 14% were SMX-responsive while 43% were cross reactive TCCs (Castrejon et al. 2010). Interestingly, only 1/40 drug-specific TCCs generated from four SMX-hypersensitive patients was SMX-specific and showed no cross-reactivity to SMX.NO (see chapter 3).

Although the full clinical implications of the mode of SMX presentation to drug-specific T-cells have not been fully explored, the ultimate outcome is T-cell activation and tissue damage. Engler et al (2004), reported that SMX.NO stimulated PBMCs from 9/10 drug naïve donors. In contrast, SMX stimulation was detected in only 3/10 donors (Engler et al. 2004). Importantly, the authors did not isolate naïve and memory T-cells; thus, the origin of drug specific T-cells are unknown. The threshold of antigen specific T-cell activation for memory T-cells is considerably lower than that required to activate naïve T-cells (Viola and Lanzavecchia 1996; Engler et al. 2004). Hence, it is possible that primary immune response may be due to SMX.NO, while SMX activates pre-existing peptide-specific T-cells.

A number of *in-vitro* experiments have been developed for the diagnosis and subsequent prevention or treatment of potential ADRs. The DC-T-cell priming

assay developed by Faulkner et al., 2012 is one such assay (Faulkner et al. 2012). It involves priming of naïve T-cells to a specific drug antigen presented by autologous dendritic cells. The primed T-cells are subsequently restimulated against test drugs using a second batch of DCs. The assay was developed using SMX.NO as a model drug antigen; however, experiments with the parent compound have thus far not been performed.

The aim of this chapter was to prime naïve T-cells to SMX and SMX.NO with a view to understanding the molecular mechanism of naïve T-cell activation by drugs as well as their protein reactive metabolites.

4.2 Aim

The molecular mechanism of SMX-hypersensitivity has been studied in detail; however, our understanding is still far from complete. A number of questions still remain unanswered. These include: (1) what conditions drive the specificity of T-cells to either the parent drug (SMX) or its reactive metabolite (SMX.NO). (2) What is the origin of SMX/SMX.NO specific TCCs and (3) why do some SMX-specific TCCs show cross reactivity with SMX.NO and others do not cross-react? We hypothesised that different T-cell populations are differentially primed by either SMX or SMX.NO.

The aims of this chapter therefore were:

- To perform DC-priming of naive T-cells with both SMX and SMX-NO.
- To generate drug-specific TCCs from primed T-cells and to characterise T-cell cross reactivity.

4.3 Methods

4.3.1 Isolation of PMBCs and separation of subsets of T-cells

Venous blood (120 ml) from 4 SMX-naïve volunteers was collected and PBMCs isolated as previously described in section 3.3.3. Approval for the study was acquired from the Liverpool local research ethics committee and informed written consent was obtained from the blood donors. $CD14^+$ monocytes and different T-cell populations were separated using magnetic beads and columns according to the manufacturer's instructions (Miltenyi Biotech; Bisley, UK). $CD14^+$ cells were positively selected from total PBMC. For isolation of naive and memory T-cells, pan negative T-cell separation was performed using an anti-T-cell antibody cocktail. $CD3^+$ cells were then subjected to positive selection for T_{reg} ($CD25^+$) and memory cells ($CD45RO^+$). Cells were frozen and stored at -150°C prior to use.

4.3.2 Lymphocyte transformation test (LTT)

LTT was performed on isolated PBMCs to assess lymphocyte proliferation following SMX or SMX.NO stimulation using protocol described in section 3.3.4.

4.3.3 T-cell priming assay

$CD14^+$ cells were cultured in medium (RPMI-1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml transferrin, 10% human AB serum, 25 mM HEPES buffer, and 2 mM L-glutamine) supplemented with GM-CSF (800 U/mL) and IL-4 (800 U/mL) under an atmosphere of 95% O_2/5% CO_2 for 7 days to generate dendritic cells. On the penultimate day, TNF-α (25 ng/mL) and LPS (1 µg/mL) were added as maturation factors. Mature dendritic cells were plated (0.8×10^5 cells per well) and co-cultured with naïve or memory $CD3^+$ T-cells (2.5×10^6 cells per well; 48 well plate) in the presence of either SMX (2mM) or SMX.NO (50 µM)

for 8 days. Figure 4.1 shows the microscopic images of immature dendritic cells, mature dendritic cells and co-culture of CD45RA naïve T-cells and mature dendritic cells (25:1). Matured DCs develop branched projections (dendrites) know to enhance antigen presentstion.

Primed T-cells (1×10^5; 200µl) were harvested and re-stimulated with autologous dendritic cells (4×10^3) in the presence of either SMX (2 mM) or SMX.NO (50 µM) and assessed for cytokine secretion as well as proliferation. After 48 hours, [^{3}H]-thymidine (0.5 µCi/well) was added to the proliferation plate. Incorporated radioactivity was counted after a further 16 hour incubation using a MicroBeta TriLux 1450 LSC β-counter (Perkin Elmer, Cambridge, UK). ELISpot was used, according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden) to visualize IFN-γ secretion.

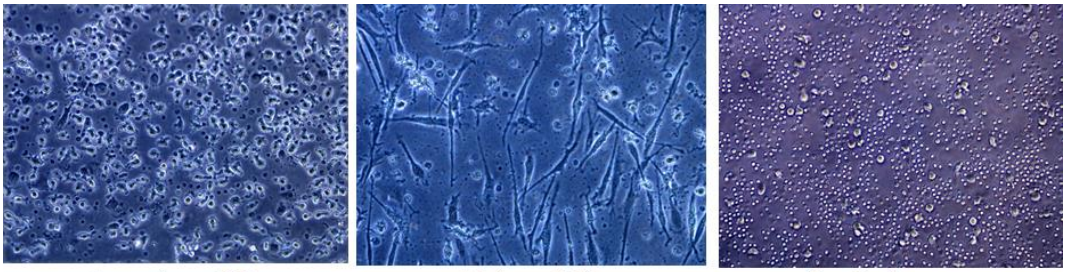


Figure 4.1-Microscopic images of immune cells in culture. (A). Immature dendritic cells cultured from $CD14^+$ cells in DC culture medium at day 6. **(B).** Mature dendritic cells following treatment of immature DCs with TNF (25 ng/ml) and LPS (1 ug/ml). **(C).** Co-culture of naïve T-cells and dendritic cells (25:1)

4.3.4 Extended priming of naïve T-cells

The extended priming assay was performed using similar conditions as reported in section 4.3.3 but the duration of priming was extended from 8 days to 10 weeks and both naïve and memory T-cell populations were primed. T-cell

cultures were restimulated with autologous PBMCs ($1.5x10^6$ cells/mL), PHA (10 μg/mL) and IL-2 (5 μg/mL) every 14 days to maintain growth and survival. T-cell culture was assessed using [^{3}H]-thymidine proliferation and IFN-ϒ secretion in the presence of either SMX or SMX.NO every 2 weeks for a total of 10 weeks.

4.3.5 T-cell cloning

T-cell cloning was performed from SMX and SMX.NO primed naïve or memory T-cells using the same protocol described in section 3.3.5.

4.3.6 Flow cytometry

TCCs were accessed for CD4 and CD8 cell surface molecules according to the protocol described in section 3.3.8.1.

4.3.7 T-cell proliferation and characterization of cytokine secretion profile

Drug specific T-cell clones were characterised using [^{3}H]-thymidine incorporation and ELISpot assays for IFN-ϒ, IL-5, IL-13 and granzyme-B as previously described in sections 3.3.9 and 3.3.10.

4.3.8 MHC restriction of SMX.NO recognition

MHC-restricted SMX.NO recognition by SMX.NO-specific TCCs was investigated according to the method described in section 3.3.11.

4.3.9 T-cell receptor V_β analyses of SMX.NO-specific TCCs

The TCR V_β usage of SMX.NO-specific TCCs was determined using the method described in section 3.3.12.

4.3.10 Statistical analysis

Mean values and standard deviations were calculated, and statistical analysis was performed using paired T tests (Sigma plot 12 software)

4.4 Results

4.4.1. Lymphocyte transformation test (LTT)

PBMCs isolated from the 4 SMX-naïve volunteers did not proliferate to graded concentrations of either SMX (0.5-2 mM) or SMX.NO (12.5-50 µM), see figure 4.2.

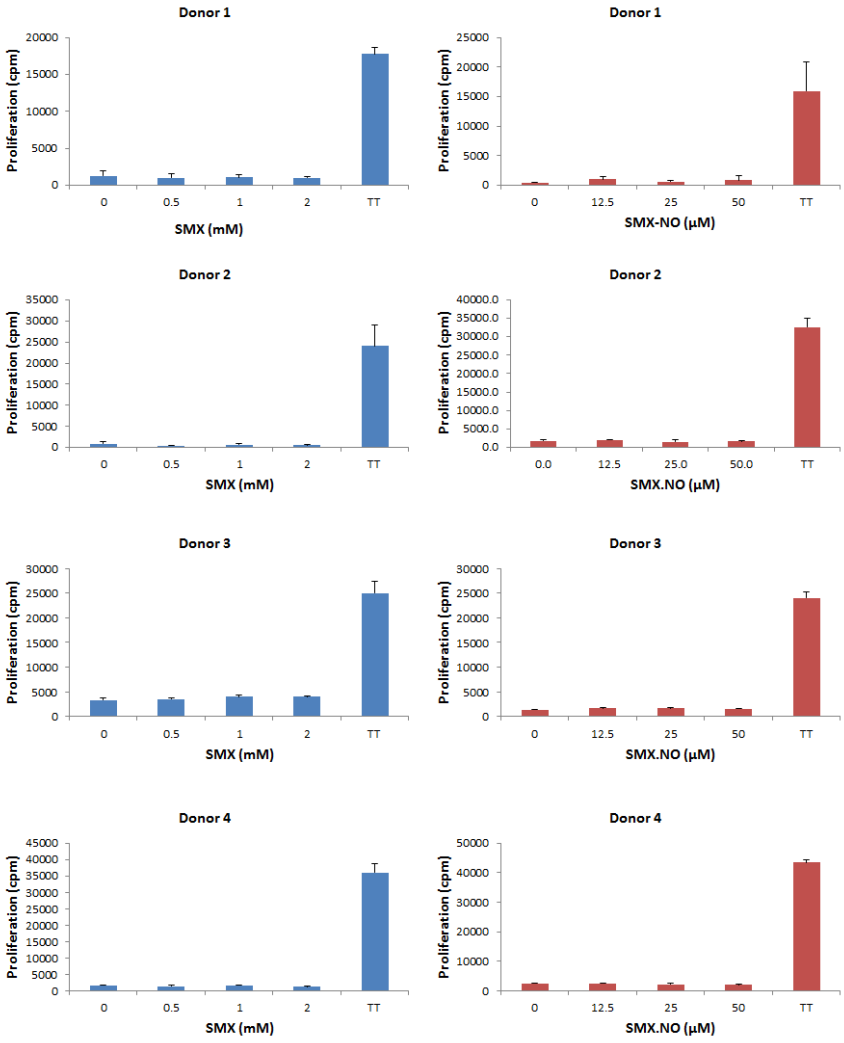


Figure 4.2-LTT assay for 4 SMX-naïve volunteers (Donor 1-4). PBMCs (1.5×10^4 cells, 100 µL) were incubated with graded concentrations of SMX (0.5-2 mM), left panel or SMX.NO (12.5-50 µM), right panel in 96-well U-bottom well plates. Tetanus toxoid was used as a positive control. Plates were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 5 days. [^{3}H]-thymidine (0.5 µCi/well) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting.

4.4.2 Characterization of DC-primed naïve T-cells

SMX or SMX.NO primed naïve T-cells were co-cultured with fresh autologous monocyte derived dendritic cells (Mo-DC) in a 25:1 ratio in the presence of either SMX (2mM) or SMX.NO (50 μM) for 48 hours. T-cell proliferation and IFN-ϒ secretion were assessed using [^{3}H]-thymidine proliferation and ELISpot assays. SMX.NO priming of naïve T-cells was observed in all 4 donors (figure 4.4 and 4.5). This is consistent with cumulative data within the group showing successful priming of naïve T-cells from 24 SMX-naïve donors to SMX.NO using readouts for ^{3}H-proliferation (16/24), IFN-ϒ secretion(24/24) and IL-13 secretion (22/24)(figure 4.3). Naïve T-cells from donor 4 were successfully primed to SMX in one experiment however; this result could not be replicated in two other repeat experiments (figure 4.4).

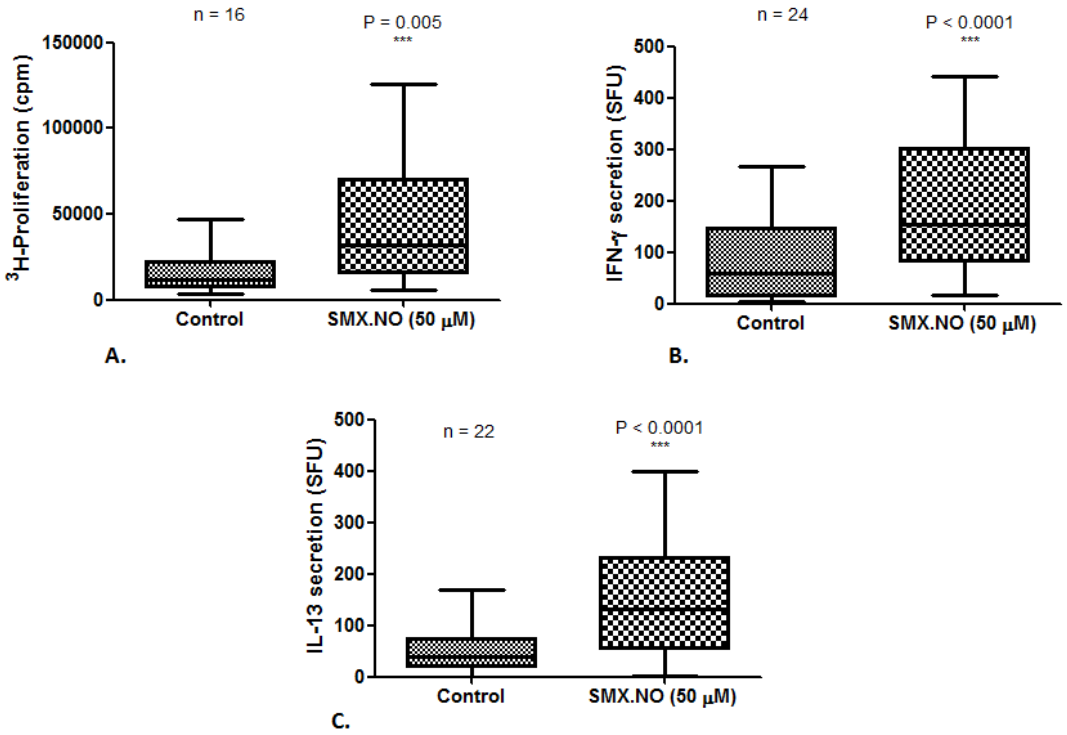


Figure 4.3 DC-priming of naïve T-cells to SMX.NO. (A) Naïve T-cells were co-cultured with Mo-DC in a 25:1 ratio in the presence of SMX.NO (50 μM) for 8 days in a 48 well plate. T-cells (1×10^5) were harvested and cultured with fresh Mo-DCs (4×10^3) in the presence of SMX.NO (50 μM) and plates incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. ^{3}H-thymidine (0.5 μCi/well) was added to each well in the final 16 hours of culture and T-cell proliferation was determined using scintillation counting. (**B and C)** ELISpot plates were coated with human either IFN-Ɣ or IL-3 antibody according to manufacturer's instruction and incubated overnight at 4°C. Drug-primed naïve T-cells (0.5×10^6) were co-cultured with Mo-DCs (4×10^3), with either SMX (2 mM) or SMX.NO (50 μM) using culture medium as negative control. Plates were then incubated at 37°C for 48 hours. The ELISpot plate was developed according to manufacturer's instruction. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader.

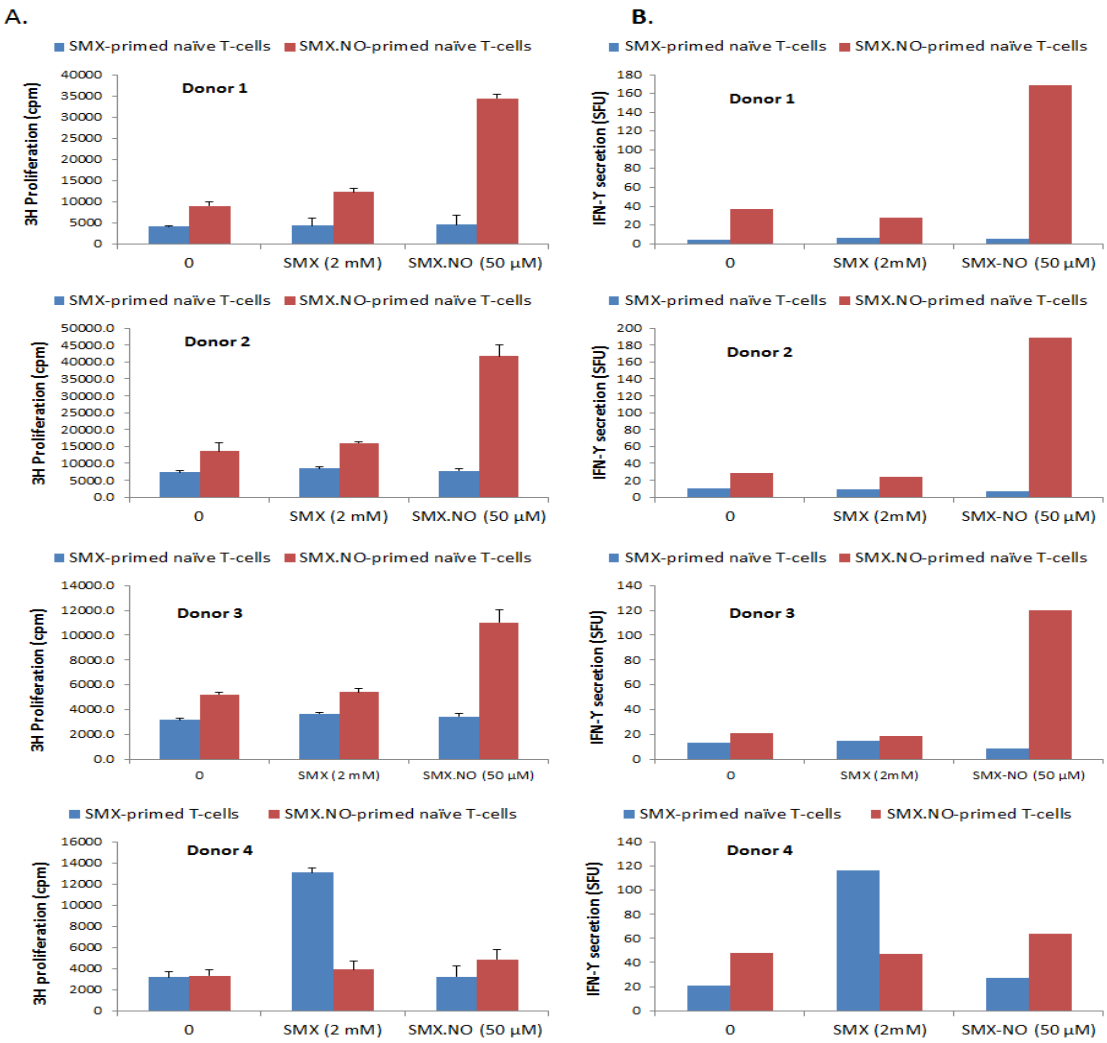


Figure 4.4-Antigen-specific T-cell responses determined by ^{3}H-thymidine proliferation and IFN-ϒ ELISpot assays. (A) Naïve T-cells were co-cultured with Mo-DC in a 25:1 ratio in the presence of either SMX (2 mM) or SMX.NO (50 µM) for 8 days in a 48 well plate. T-cells (1×10^{5}) were co-cultured with fresh Mo-DCs (4×10^{3}) in the presence of either SMX (2 mM) or SMX.NO (50 µM) and plates incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. ^{3}H-thymidine (0.5 µCi/well) was added to each well in the final 16 hours of culture. T-cell proliferation was determined using scintillation counting. **(B).** ELISpot plates were coated with human IFN-ϒ antibody according to manufacturer's instruction and incubated overnight at 4°C. Drug-primed naïve T-cells (0.5×10^{6}) were co-cultured with either Mo-DCs (4×10^{3}) with either SMX (2 mM) or SMX.NO (50 µM) using culture medium as negative control. Plates were then incubated at 37°C for 48 hours. The ELISpot plate was developed according to manufacturer's instruction. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader. Blue bars represent SMX-primed naïve T-cells while red bars represent SMX.NO-primed naïve T-cells.

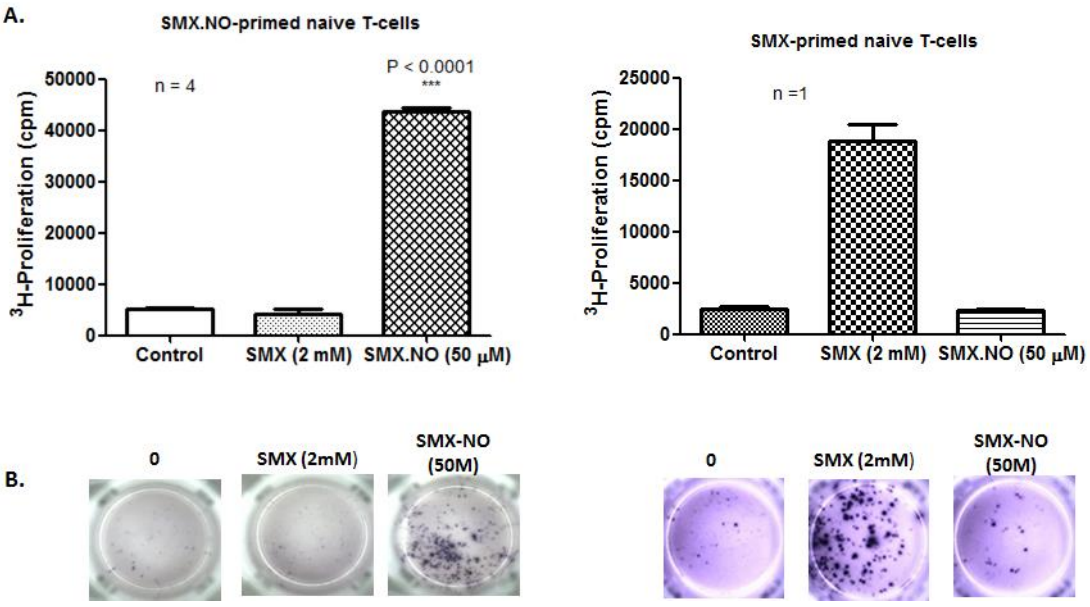


Figure 4.5-Proliferation and IFN-ϒ secretion profile of SMX/SMX.NO-primed naïve T-cells from four SMX-naïve donors. (A) Naïve T-cells were co-cultured with Mo-DC in a 25:1 ratio in the presence of SMX (2 mM) or SMX.NO (50 μM) for 8 days in 96-well U-bottom plates. T-cells (1×10^5) were harvested and cultured with fresh Mo-DCs (4×10^3) with either SMX (2 mM) or SMX.NO (50 μM) and plates incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. ^{3}H-thymidine (0.5 μCi/well) was added to each well in the final 16 hours of culture. T-cell proliferation was evaluated using scintillation counting. **(B)** ELISpot plates were coated with human IFN-ϒ antibody according to manufacturer's instruction and incubated overnight at 4°C. SMX-primed naïve T-cells (0.5×10^6) were co-cultured with either Mo-DCs (4×10^3) in the presence of either SMX (2 mM) or SMX.NO (50 μM) using culture medium as negative control. Plates were then incubated at 37°C for 48 hours. The ELISpot plate was developed according to manufacturer's instruction. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader

4.4.3 Effect of extended exposure to SMX on naïve T-cell priming.

Naïve T-cells primed with either SMX or SMX.NO was maintained in culture for a total period of 10 weeks. ^{3}H-thymidine proliferation and IFN-ϒ ELISpot assays were used to evaluate T-cell activation every 2 weeks. Prolonged exposure of naïve T-cells to either SMX or SMX.NO did not enhance the priming of naïve T-cells to SMX (figure 4.6).

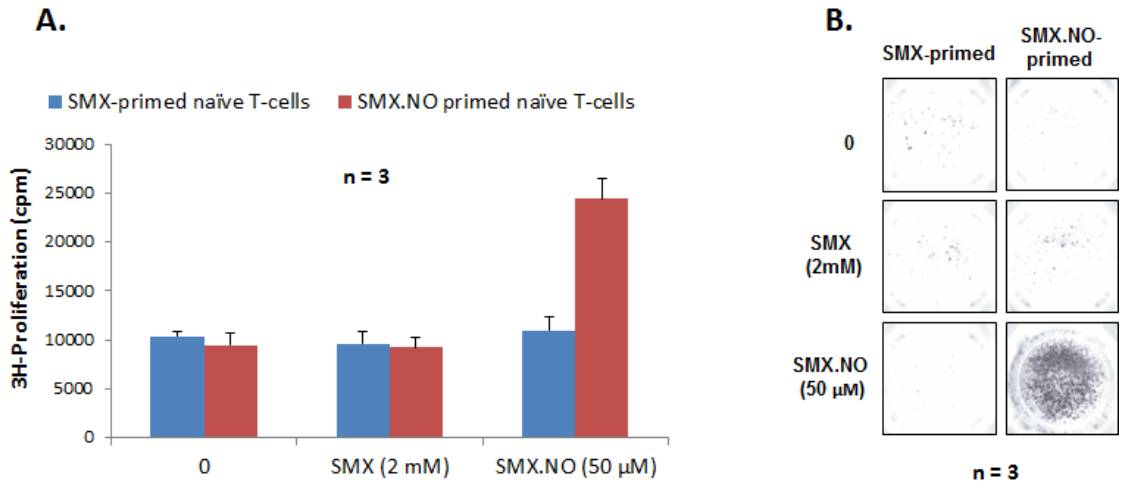


Figure 4.6-T-cell proliferation and IFN-ϒ secretion after extended priming of naïve T-cells to either SMX or SMX.NO. (A). Naïve T-cells were co-cultured with Mo-DC in a 25:1 ratio in the presence of either SMX (2 mM) or SMX.NO (50 µM) for 10 weeks in a 48 well plate. Every 2 weeks, T-cells (1×10^5) were co-cultured with fresh Mo-DCs (4×10^3) in the presence of either SMX (2 mM) or SMX.NO (50 µM) and plates incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. ^{3}H-thymidine (0.5 µCi/well) was added to each well in the final 16 hours of culture. T-cell proliferation was determined using scintillation counting. Blue bars represent SMX-primed naïve T-cells while red bars represent SMX.NO-primed naïve T-cells. **(B).** ELISpot plates were coated with human IFN-ϒ antibody according to manufacturer's instruction and incubated overnight at 4°C. Drug-primed naïve T-cells (0.5×10^6) were co-cultured with either Mo-DCs (4×10^3) with either SMX (2 mM) or SMX.NO (50 µM) using culture medium as negative control. Plates were then incubated at 37°C for 48 hours. The ELISpot plate was developed according to manufacturer's instruction. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader.

4.4.4 Characterization of drug-specific TCCs generated from DC-primed naïve T-cells

A total of 168 T-cell clones were generated and tested from SMX- and SMX.NO-primed naïve T-cells (1 volunteer). Ten of the TCCs tested were SMX.NO responsive but showed no cross reactivity with SMX (figure 4.7). Furthermore we investigated the effect of extended SMX/SMX.NO priming of either naïve T-cells and memory T-cells. Interestingly, preliminary data suggests that naïve T-cells were primed to SMX.NO while memory T-cells were primed to SMX (figure 4.8). All SMX.NO responsive TCCs generated from the 8 day priming assay expressed the CD4 cell surface marker (figures 4.9).

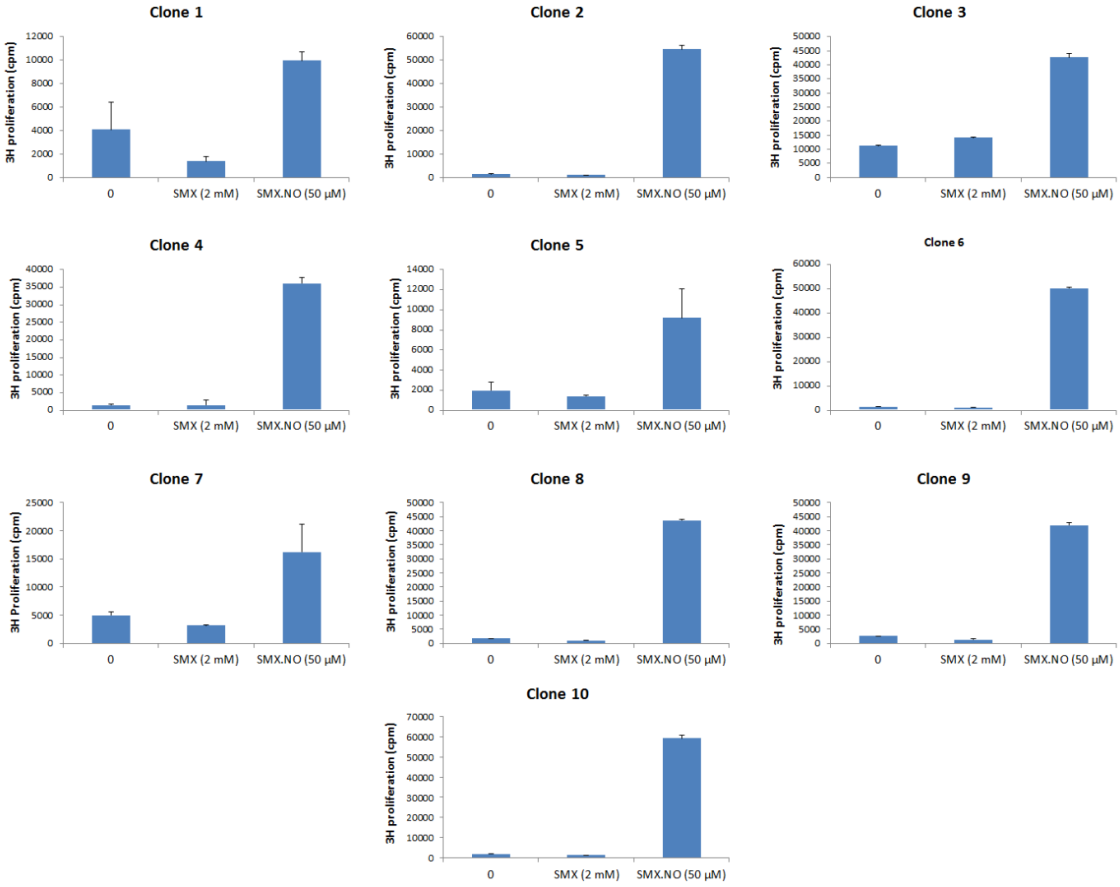


Figure 4.7-Proliferation and cross reactivity of SMX.NO-specific TCCs. T-cell clones (5×10^4 cells, 50 µL) were co-incubated with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells, 50 µL) in the presence of either SMX (2 mM) or SMX.NO (50 µM) in a 96-well U-bottom microplate using T-lymphocyte culture medium as negative control. The plates were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. [^{3}H]-thymidine (0.5 µCi/mL) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting. Result shows 10 SMX.NO-specific TCCs generated from drug-primed naïve T-cells.

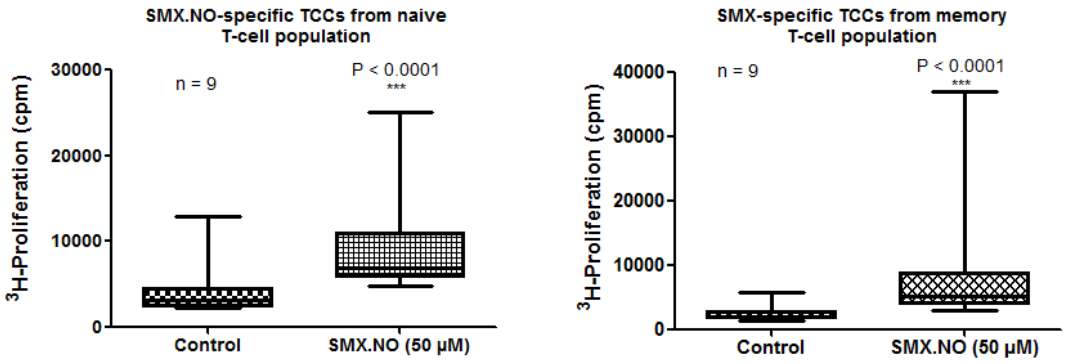


Figure 4.8 Antigen specificity of TCCs generated from either naïve or memory T-cell populations. Naïve and memory T-cell populations were primed with either SMX or SMX.NO for 10 weeks. TCCs were then generated from primed T-cells according to the method described. ^{3}H-thymidine incorporation assay was used to assess antigen specificity.

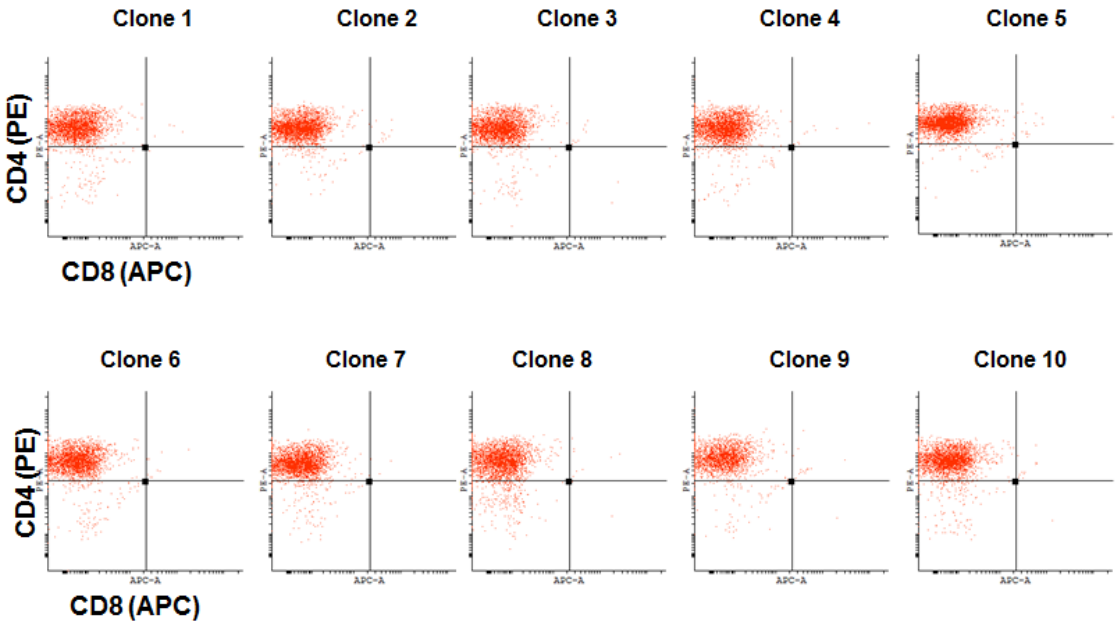


Figure 4.9-CD4 and CD8 phenotyping. TCC suspensions (50 µL) were incubated with both CD4-PE and CD8-APC antibodies for 20 minutes at 4°C. Cells were washed and signals acquired by flow cytometry and analysed using cyflogic.

TCCs generated from naïve T-cells primed for 8 days secreted significant amounts of IFN-ϒ, IL-5, IL-13 and granzyme-B in response to SMX.NO stimulation (figure 4.10A). MHC restriction using anti-HLA class I/II antibodies revealed an HLA-DP-restricted SMX.NO presentation to drug-specific TCCs (figure 4.9B). SMX.NO-specific TCCs expressed two different repertoires of TCRV_β namely, V_β2 (86%) and V_β13.1 (14%) as illustrated in figure 4.10C and D. Further experiments are ongoing to characterise TCCs generated from extended priming of naïve and memory T-cell populations.

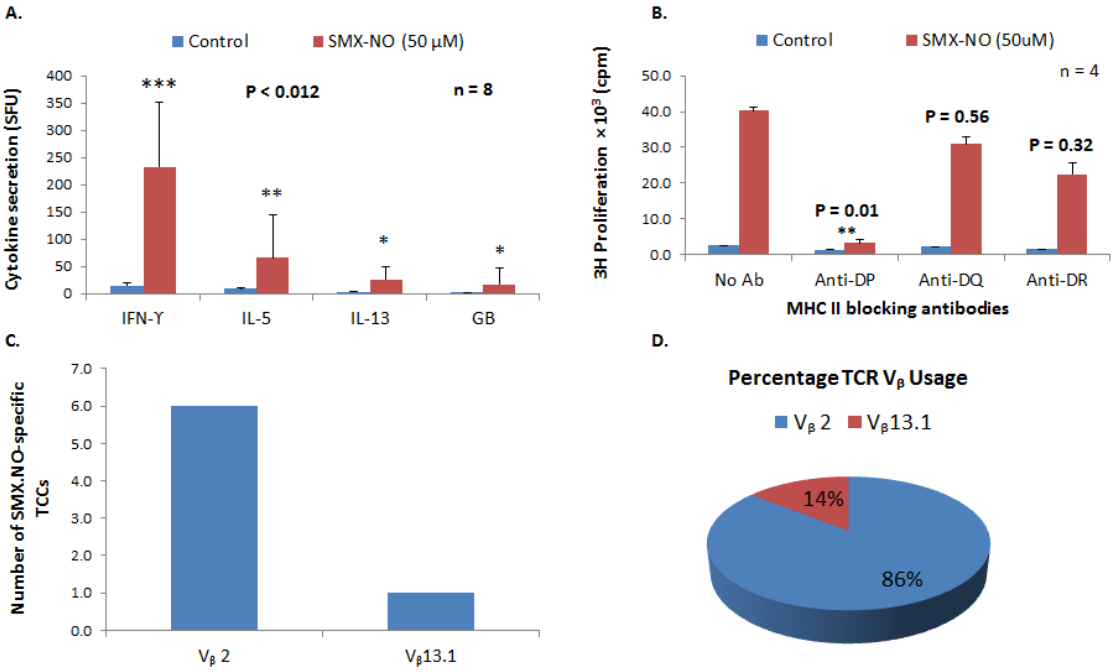


Figure 4.10-Characterization of SMX.NO-specific TCCs. (A) Cytokine secretion profiles of 8 representative drug-specific TCCs. ELISpot plates were pre-coated with human IFN-Ɣ, IL-5, IL-13 and granzyme-B antibodies according to manufacturer's instruction and incubated overnight at 4°C. T-cell clones (5×10^4, 50 µL) were co-incubated with irradiated autologous EBV-transformed B-cells (1×10^4, 50 µL) and SMX.NO (50 µM) using culture medium as negative control. The plates were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. The ELISpot plates were developed according to manufacturer's instruction. Wells were then left to air dry and spots visualised and counted using an AID ELISpot reader. **(B) HLA-DP, -DQ and –DR restricted SMX.NO presentation to TCCs**. Irradiated autologous EBV-transformed B-cells (1×10^4 cells, 50 µL) were pre-incubated with anti-HLA-DP, -DQ or –DR blocking antibodies (5 µg/mL) for 30 minutes at 37°C. APCs were then co-incubated with drug-specific TCCs (5×10^4 cells, 50 µL) in the presence or absence of SMX.NO (50 µM) in a 96-well U-bottom microplate using culture medium as negative control. The plate was incubated at 37°C for 48 hours. [^{3}H]-thymidine (0.5 µCi) was added to each well for the final 16 hours of incubation and T-lymphocyte proliferation evaluated using scintillation counting. **(C and D)** Graphical representation of TCR V_β usage in SMX.NO-specific TCCs and the percentage TCR V_β expression of 7 representative SMX.NO-specific TCCs.

4.5 Discussion

Idiosyncratic drug hypersensitivity reactions are problematic because they can result in serious morbidity and mortality. These reactions are also difficult to predict during the pre-clinical stages of drug development. Idiosyncratic drug hypersensitivity reactions only become evident during the wide spread clinical use of the 'culprit drug'. They occur in a small percentage of the population and are thought to be associated with a combination of environmental, genetic, chemical properties of the drug involved and pathophysiological factors, hence their unpredictable nature. This is made even more complicated because the combination of risk factors for drug hypersensitivity reactions may differ depending on the 'offending drug' (Knowles et al. 2002; Macy 2004). Presently, it is difficult to predict what drug molecules will cause serious hypersensitivity reactions and in which patients. Therefore, an in-depth understanding of the pathomechanism of these reactions will likely increase their predictability; hence the need for *in-vitro* and *in-vivo* assays that can predict the immunogenic potential of a new drug.

At the moment, very few animal models of drug hypersensitivity reactions exist (Weiss et al. 1978; Stein et al. 1980; Nierkens and Pieters 2005; Uetrecht 2005). The limitation of developing an animal model for every new drug is in the number of animals that will be required for such studies but also in aligning the pathomechanism of drug hypersensitivity reactions in animal models to that in humans (Uetrecht 2005). Furthermore, the increasing association of many idiosyncratic drug hypersensitivity reactions with HLAs complicates the prospects for ideal animal models of drug hypersensitivity reactions. Hence, *in-vitro* models based on relevant human cells are the most likely to generate

mechanistic data that enhance our understanding of chemical and immunological mechanisms.

Although many drug hypersensitivity reactions can be attributed to protein-reactive drug metabolites (Uetrecht 1999; Uetrecht 2005), some inert drugs are also implicated (Zanni et al. 1997; Schnyder et al. 2000; Elsheikh et al. 2010). The *in-vitro* priming assay developed by Faulkner et al., (2012) explored the possibility of predicting the immunostimulatory capability of drug naïve donor to a given drug (Faulkner et al. 2012). The authors reported that naïve T-cells isolated from 5 SMX-naïve volunteers were successfully primed to the reactive metabolite, SMX.NO. In this study, we attempted to replicate these data by priming naïve T-cells isolated from 4 donors to SMX.NO. Moreover, we explored whether the parent drug (SMX) could activate naïve T-cells.

The LTT was negative for both SMX and SMX.NO in all the donors used for this study. This suggested they do not have SMX or SMX.NO-specific T-cells circulating in their peripheral blood. The priming assay involves the initial *in-vitro* co-culture of naïve T-cells and dendritic cells with either SMX or SMX.NO for 8 days followed by stimulation of the T-cells and assessment of antigen specificity using readouts for T-cell proliferation and IFN-ϒ secretion. Naïve T-cells were successfully primed to SMX.NO in all 4 drug-naïve donors. Interestingly, initial experiments suggested that naïve T-cells isolated from donor 4 was primed to SMX (n =1). Although no data exist at the moment, we speculate that genetic factors, most likely donor HLA is responsible for the selective SMX-priming of naïve T-cells observed in donor 4.

To confirm priming of naïve T-cells to either SMX or SMX.NO, drug-specific TCCs were generated from DC-primed naïve T-cells (8 days priming). Interestingly, all the clones generated were SMX.NO responsive and expressed the CD4 cell surface molecule. This is consistent with TCCs generated from PBMCs isolated from SMX-hypersensitive patients (chapter 3). Furthermore, MHC restriction assay revealed an HLA-DP restricted SMX.NO recognition by drug-specific TCCs. This contrasts the HLA-DQ restricted SMX.NO recognition observed for SMX.NO-specific TCCs generated from hypersensitive patients (chapter 3). Paradoxically, the majority of the SMX.NO-specific TCCs generated from either SMX-hypersensitive patients or SMX-naïve donor expressed the TCR $V_{\beta}2$, 69% and 86%, respectively (see chapter 3). It is possible that the pathophysiological state of patients may impact on HLA restriction observed in SMX-hypersensitive patients. Healthy volunteer cohorts provide an excellent experimental resource for research into the pathomechanism of drug hypersensitivity but data generated must be interpreted with caution.

To investigate whether the duration of naïve T-cell priming has any effect on the threshold of T-cell activation, we performed an extended priming assay. This involved an increase in the initial co-culture period of naïve T-cells, DCs and either SMX or SMX.NO from 8 days to 10 weeks. ^{3}H-thymidine proliferation and IFN-ϒ data suggested that an extend duration of naïve T-cell priming to either SMX or SMX.NO did not lower the threshold of activation for SMX-primed naïve T-cells. Furthermore, in this study, all 10 drug-specific T-cell clones generated after the DC-priming of naïve T-cells isolated from 1 SMX-naïve donor were SMX.NO responsive and showed no cross-reactivity with SMX. The variable

distribution of drug-specific TCRs suggests that factors other than the chemical properties of either SMX or its nitroso metabolite may be important determinants of specificity. Although SMX and SMX.NO have similar chemical structures, the reason behind why most naïve T-cells from SMX-naïve volunteers are activated by SMX.NO and a few by SMX remains enigmatic. The high degree of protein reactivity may be responsible but this is not sufficient to explain why some SMX.NO-specific T-cells show cross-reactivity with SMX.

Very recent data generated within the lab suggests that TCCs generated from naïve T-cell populations were SMX.NO-responsive while those generated from the memory T-cell population were SMX-specific. Further experiments including cross reactivity, MHC restriction, CD4/CD8 phenotyping, cytokine profiling are ongoing to delineate the molecular mechanisms involved in this observed selective priming of different T-cell subsets to either SMX or SMX.NO.

Chapter 5: Negative regulation by PD-L1 during drug-specific priming of IL-22 secreting T-cells and the influence of PD-1 on effector T-cell

Contents

5.1 Introduction

Immunological drug reactions represent a major clinical problem because of their severity and unpredictable nature. In recent years, genome-wide association studies have identified specific HLA alleles as important susceptibility factors for certain reactions (Phillips et al. 2011; Daly 2012). Drug antigen-specific $CD4^+$ and/or $CD8^+$ T-cell responses are detectable in blood/tissue of patients presenting with mild and severe forms of skin injury (Nassif et al. 2002; Castrejon et al. 2010) and liver injury (Monshi et al. 2013), and are therefore believed to participate in the disease pathogenesis.

For a limited number of drugs, the drug-derived antigen has been shown to interact specifically with the protein encoded by the HLA risk allele to activate T-cells (Monshi et al. 2013; Yun et al. 2014). However, one must emphasize that (1) strong HLA associations have not been identified for most forms of drug hypersensitivity and (2) the majority of individuals who carry known HLA risk alleles do not develop clinically relevant immunological reactions when exposed to a culprit drug (Daly et al. 2009). Thus, there is a need to characterize the immunological parameters that are superimposed on HLA-restricted T-cell activation to determine why particular individuals develop drug hypersensitivity. Infections, especially reactivation of the herpes virus family have been put forward as an additional risk factor (Descamps et al. 2001; Picard et al. 2010). Virus infection alone however does not fully explain the unpredictable nature of drug hypersensitivity.

Thus, this chapter focuses on the model drug hapten nitroso-sulfamethoxazole (SMX.NO) to investigate whether the programmed death (PD) pathway regulates the drug-specific priming of naïve T-cells. SMX.NO has been shown

previously to activate $CD4^+$ and $CD8^+$ T-cells isolated from patients presenting with sulfamethoxazole induced cutaneous injury (Schnyder et al. 2000; Engler et al. 2004; Castrejon et al. 2010; Faulkner et al. 2012).

PD-1 (CD279) is an immune inhibitory receptor which belongs to the CD28 superfamily of immune regulators (Freeman et al. 2006; Sharpe et al. 2007). The CD28 superfamily of proteins control the fine balance between immune response and immune tolerance (Chen 2004; Greenwald et al. 2005; Okazaki and Honjo 2006). PD-1 is expressed on activated T-cells and known to modulate an inhibitory pathway which maintains peripheral tolerance (Nishimura et al. 1998; Chemnitz et al. 2004; Fife et al. 2009). The critical inhibitory role of PD-1 has been demonstrated in the development of several forms of autoimmune disease through the use of $PD\text{-}1^{-/-}$ mice (Nishimura et al. 1998; Okazaki and Honjo 2006). A number of studies have reported PD-1 expression on DCs and macrophages (Huang et al. 2009; Yao et al. 2009). Expression of PD-1 occurs during thymic expansion and is activated through antigen receptor signalling and cytokine activity (Francisco et al. 2010).

Activation of the PD-1 receptor, which is transiently expressed on activated T-cells leads to clustering between T-cell receptors and the phosphatase SHP2, and dephosphorylation of T-cell receptor signalling (Duraiswamy et al. 2011). As such, PD-1 signalling represents an effective mechanism to suppress antigen-specific T-cell responses upon continuous antigen stimulation (Sharpe and Freeman 2002; Chen 2004; Okazaki and Honjo 2006). PD-1 has two ligands; PD-L1 (CD274) and PD-L2 (CD273) (Freeman et al. 2000; Latchman et al. 2001; Keir et al. 2008). PD-1/PD-L interactions inhibit T-cell proliferation and cytokine secretion (Freeman et al. 2000; Latchman et al. 2001). Cytolytic

function and T-cell survival are also significantly impaired (Keir et al. 2008; Riley 2009). PD-1 ligation interferes with T-cell proliferation, cytokine secretion, cytolytic function and survival by either directly inhibiting CD28 induced activation signals or indirectly by obstructing IL-2 secretion (Riley 2009; Francisco et al. 2010). Figure 5.1 illustrates the different proteins important in the PD-1 signalling pathway. PD-1 is a type I membrane protein consisting of 288 amino acid residues (Riley 2009). The protein is made up of an extracellular domain and an intracellular tail, both connected by a transmembrane. The intracellular tail consists of two phosphorylation sites, each located in an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif (Ishida et al. 1992; Blank and Mackensen 2007). PD-1 ligation with its ligands is thought to block Akt phosphorylation by inhibiting the CD28-mediated activation of PI3k (upstream of Akt). The immunoreceptor tyrosine-based inhibitory motif is responsible for the PD-1 inhibition of the PI3K/Akt pathway. Furthermore, PD-1 signalling inhibits the CD28 mediated stimulation of PI3K and Akt by engaging SHP2 (Parry et al. 2005; Yokosuka et al. 2012). SHP2 also inhibits the phosphorylation of ZAP70 (figure 5.1).

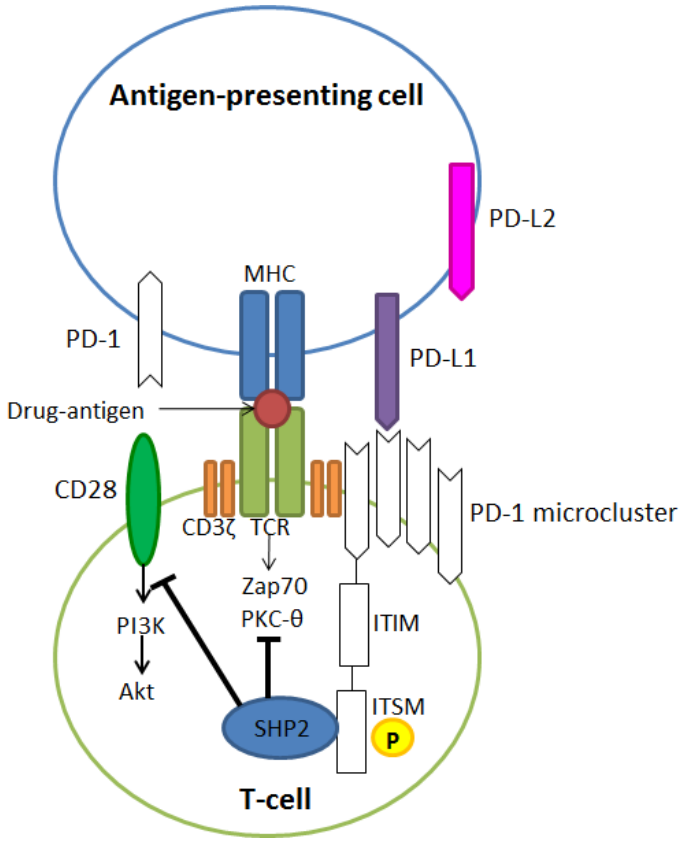


Figure 5.1–PD-1 signalling pathway. Schematic representation of T-cell interaction with antigen presenting cell showing PD-1, PD-L1, PD-L2 and other proteins involved in the PD-1 signalling pathway. PD-1: programmed cell death 1, PD-L: programmed death ligand, MHC: major histocompatibility complex, TCR: T-cell receptor, SHP2: Src homology 2 domain-containing tyrosine phosphatase 2, ITIM: immunoreceptor tyrosine-based inhibition motif, ZAP 70: Zeta-chain-associated protein kinase 70, PKC: Protein kinase C, PI3K: Phosphatidylinositide 3-kinases, Akt: Serine threonine kinase.

PD-L2 has a greater binding affinity for PD-1 compared to PD-L1 as B7-1 is a supplementary receptor for PD-L1 (Butte et al. 2007). While PD-L1 is expressed on a variety of immune cells, PD-L2 expression is limited to dendritic cells, bone-marrow-derived mast-cells and activated macrophages (Ishida et al. 2002; Yamazaki et al. 2002; Yokosuka et al. 2012). PD-L1 has a higher expression in humans than PD-L2 but its ability to inhibit IL-2 secretion and effector cytokine production overlap (Keir et al. 2006). Regulatory T-cells have been reported to express both PD-1 and PD-L1 (Keir et al. 2007). The co-expression of PD-L1 and

PD-L2 on mast-cells and recent findings showing that mast-cells are involved in regulatory T-cell dependent peripheral tolerance has generated more interest in the PD-1-PD-L pathway (Lu et al. 2006; Nakae et al. 2006; Keir et al. 2007). Although PD-1 has been classified as a marker of cell exhaustion, (Dyavar Shetty et al. 2012; Zinselmeyer et al. 2013) recent studies from independent laboratories describe an alternative perspective (Dong et al. 1999; Tseng et al. 2001). Blockade of PD-1-PD-L signalling has been reported to restore function of exhausted T-cells (Barber et al. 2006; Boni et al. 2007; Radziewicz et al. 2007).

Therapeutic potential of blocking the PD-1/PD-L1 pathway has been reported in cancer therapy where high expression of PD-L1 is consistent with poor prognosis (Brahmer et al. 2010; Duraiswamy et al. 2011; Topalian et al. 2012). Tumour cells utilise this inhibitory pathway to elude clearance by the host's immune system (Dong et al. 2002; Thompson et al. 2007). Duraiswamy et al. showed that most PD-1^{high} human $CD8^{+}$ T-cells are effector memory cells rather than exhausted cells (Duraiswamy et al. 2011). Zelinskyy et al showed that although virus-specific $CD8^{+}$ T-cells upregulate PD-1 expression during acute infection, the majority of PD-1^{high} cells were highly cytotoxic and controlled virus replication (Barber et al. 2006; Zelinskyy et al. 2011).

Finally, Reiley et al. showed that PD-1^{high} $CD4^{+}$ T-cells were highly proliferative and appeared to maintain effector T-cell responses during chronic infection (Reiley et al. 2010). Consequently, in the present study T-cell clones were generated from SMX-hypersensitive patient PBMCs and healthy drug naïve donors following *in vitro* priming to characterize the cytokine signatures(s) of

SMX.NO-specific T-cells and study whether PD-1 expression/signalling governs the differentiation of T-cells into effector/helper subsets. Antigen-specific T-cell responses were measured using readouts for proliferation, cytokine secretion and cell phenotype. Antigen-specific responses from *in vitro* primed T-cell clones and clones from hypersensitive patients were measured and correlated with PD-1 expression. We therefore hypothesised that co-inhibitory pathways may play a crucial role in naïve T-cell priming to drug antigens.

5.2 Aims

The aim of this chapter was:

- To investigate the role of PD-1/PD-L1 signalling in the activation and differentiation of drug specific effector T-cells

5.3 Clinical details of patients

Clinical details of sulfamethoxazole hypersensitive patients, origin, phenotype and specificity of the T-cell clones are represented in table 5.1 below.

Table 5.1-Clinical details of the hypersensitive patients, origin, phenotype and specificity of the T-cell clones

Patient ID	Clinical details (n)	Clones tested (n)	SMX-NO specific clone	CD Phenotype (%)		Proliferation (cpm)	
				CD4+	CD8+	Control	SMX-NO(50uM)
1:Female, Age 30	Maculopapular rash day 2 of treatment; 7 years since reaction	336	20	96.6	3.3	6840 ± 7406	14837 ± 13224
2: Male, Age 23	Maculopapular rash day 10 of treatment; 20 years since reaction	152	12	100	_	1849 ± 1659	5086 ± 3649
3: Female, Age 34	Maculopapular rash day 6 of treatment; 8 years since reaction	216	5	100	_	1058 ± 203	2740 ± 469
4: Female, Age 26	Periorbital oedema day 3 of treatment; 6 years since reaction	240	2	100	_	1584 ± 493	7613 ± 5294
5: Female, Age 25	Maculopapular rash day 4 of treatment; 5 years since reaction	394	29	100	_	3899 ± 5522	23970 ± 18651

5.4 Methods

5.4.1 Chemicals and reagents

Human AB serum was acquired from Innovative Research (Michigan, USA). Foetal bovine serum (FBS) was bought from Invitrogen, Paisley, UK. Interferon-γ, interleukin-13, interleukin-5, granzyme B and perforin ELISpot kits including antibodies and substrate solution were purchased from Mabtech (Stockholm, Sweden). The Fas ligand ELISpot kit was obtained from Abcam (Cambridge, UK). CD4-APC and CD8-PE antibodies were purchased from BD Biosciences, Oxford, UK. Recombinant human interleukin-2 (rhIL-2) was bought from Peprotech, London, UK. Multisort bead separation kits were bought from Miltenyi Biotec, Surrey, UK. Sulfamethoxazole was obtained from Sigma-Aldrich (Gillingham, Dorset, UK). Human interleukin-22 (rhIL-22) was obtained from MABTECH (Nacka strand, Sweden). Lymphoprep (Axis-shield, Dundee), Tritiated [^{3}H]-methyl thymidine was purchased from Moravek (California, USA). Anti-human CCR4 and anti-human CCR10 chemokines were obtained from R&D systems (Minneapolis, USA). Transwell plates were purchased from Corning Incorporated (Corning, USA). DMSO was supplied by Sigma-Aldrich (Dorset, UK). Purified anti-human CD273 (B7-DC, PD-L2) and purified anti-human CD 274 (B7-H1, PD-L1) were purchased from Biolegend (Cambridge, UK). Recombinant human CCL17 and CCL27 were obtained from R&D systems (Minneapolis, USA). Human interleukin-4 and recombinant human GM-CSF were supplied by Peprotech (London, UK).

5.4.2 Isolation of lymphocytes from patients and volunteers blood

Blood (120 ml) was collected from drug naive donors for lymphocyte isolation and subsequent T-cell priming. Blood (50 mL) was also collected from 5 SMX-hypersensitive patients for cloning. Table 5.1 describes the clinical features of the adverse reactions for each patient. Approval for the study was acquired from the Liverpool local research ethics committee and informed written consent was obtained. PBMCs were isolated according to method previously described in section 2.3.3.

5.4.3 Cell separation

PBMCs were isolated by density gradient separation as described in section 3.3.3. $CD14^+$ monocytes and different T-cell populations were separated using magnetic beads and columns according to the manufacturer's instructions (Miltenyi Biotech; Bisley, UK). $CD14^+$ cells were positively selected from the total PBMC population. For isolation of naïve and memory T-cells, pan negative T-cell separation was performed using an anti-T-cell antibody cocktail. $CD3^+$ cells were then subjected to positive selection for T_{reg} ($CD25^+$) and memory cells ($CD45RO^+$). Cells were frozen and stored at -150°C prior to use.

5.4.4 T cell priming assay

$CD14^+$ cells were cultured in medium (RPMI-1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml transferrin, 10% human AB serum, 25 mM HEPES buffer, and 2 mM L-glutamine) supplemented with GM-CSF and IL-4 under an atmosphere of 95% O_2/5% CO_2 for 7 days to generate dendritic cells. On the penultimate day, 25 ng/ml TNFα and 1 µg/ml LPS were added as maturation

factors. Dendritic cell phenotype (CD40, CD80, CD86, PD-L2 and MHC class II) was characterised by flow cytometry.

Mature dendritic cells were plated (0.8×10^5 cells per well) and cultured with naive $CD3^+$ T-cells (2.5×10^6 cells per well; 24 well plated total volume 1.5 ml) and SMX.NO (50 μM) for 8 days. Anti-PD-L1 and/or PD-L2 antibodies (10 μg/ml) were added to certain wells. Where indicated, TGF-β (5ng/ml), IL-1β (10 ng/ml) and IL-23 (20 ng/ml) or TNFα (50ng/ml) and IL-6 (20 ng/ml) were added to the cultures to induce the differentiation of Th17 and Th22 cells, respectively.

Primed T-cells (1×10^5; 200μl) were harvested and re-stimulated with autologous dendritic cells (4×10^3) and SMX.NO (5-50 μM) and assessed for cytokine secretion as well as proliferation. After 48 hours, [^{3}H]-thymidine (0.5 μCi/well) was added to the proliferation plate. Incorporated radioactivity was counted after a further 16 hours incubation using a MicroBeta TriLux 1450 LSC β-counter (Perkin Elmer, Cambridge, UK). Proliferation was also assessed using CSFE-labelled cells according to our recently published protocol (Faulkner et al. 2012). ELISpot analysis was used, according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden) to visualize secreted cytokines (IFN-γ, IL-13, granzyme-B, IL-17 and IL-22). Cell phenotype during priming and following restimulation was assessed by staining with CD3-APC, CD4-APC, CD8-PE, CD45RA-FITC, CD45RO-PerCP-Cy5.6 and/or PD-1-PE (CD279) antibodies.

5.4.5 T cell cloning

Primed T-cells were cloned directly by serial dilution and repetitive mitogen-driven expansion using previously described methods (Zanni et al. 1997).

5.4.5.1 Generation of drug-specific T-cell clones and characterization of cytokine secretion profiles

Drug-specific T-cell clones were generated from PBMCs isolated from hypersensitive patients and one SMX-naïve volunteer according to the method described in section 3.3.5. Dose-dependent proliferative responses (± PD-L1 block; SMX.NO [5-50 μM]) and the profile of secreted cytokines (IFN-γ, IL-5, IL-13, granzyme-B, FasL, perforin, IL-17 and IL-22 ELISpot) were then measured. Cell phenotyping was performed by flow cytometry using CD4-FITC, CD8-PE and PD-1-PE, CCR4-PE, CCR10-PE and CLA-FITC antibodies.

Twenty-four well transwell chambers with 5-μm pores were used to measure chemotaxis. T-cells (0.1×10^5; n=4 clones) were placed in the upper chambers. CCL17/CCL27 (ligand for CCR4 and CCR10 respectively) was placed in the lower wells and the cells were incubated for 0.5-24 hours. Cells migrating to the lower chamber were counted using a hemocytometer.

5.5.5.2 Generation of autologous antigen presenting cells

EBV-transformed B-cells used as antigen presenting cells in all the experiments involving drug-specific TCCs were generated according to the method described in section 2.3.5.

5.5.5.3 Flow cytometry

Cells were acquired using a FACS Canto II (BD Biosciences) and data analyzed by Cyflogic. For CFSE analysis, a minimum of 50,000 lymphocytes were acquired using forward scatter/side scatter characteristics.

5.5 Statistical analysis

Mean values and standard deviations were calculated, and statistical analysis was performed using paired T-test (Sigma plot 12 software).

5.6 Results

5.6.1 PD-L1 block enhances the priming of naïve T-cells against drug derived antigens

For *in vitro* priming, naïve CD3+ T-cells from healthy donors were co-cultured with autologous dendritic cells in the presence of SMX.NO (± PD-L1/PD-L2 block). Upon re-stimulation, dose-dependent antigen-specific proliferation was clearly detectable (figure 5.2A). Inclusion of PD-L1 blockade markedly enhanced the proliferative response ($P<0.05$; at each SMX.NO concentration). PD-L2 block however gave proliferative responses comparable to those without PD-ligand block (figure 5.2A). Blockade of PD-L1 and PD-L2 together produced enhanced proliferation compared to medium alone ($P<0.05$), but less so than for PD-L1 block. This data was unexpected and maybe due to incompatibilities in combining two different monoclonal antibodies or technical artefact of this experiment. This observation warrants further investigation. Additional SMX.NO priming experiments were performed using IFN-γ and granzyme-B secretion as readouts. IFN-γ (figure 5.2B) and granzyme-B (figure 5.2C) were released from SMX.NO primed cells following restimulation and the response was enhanced when the anti-PD-L1 antibody was included in the co-culture.

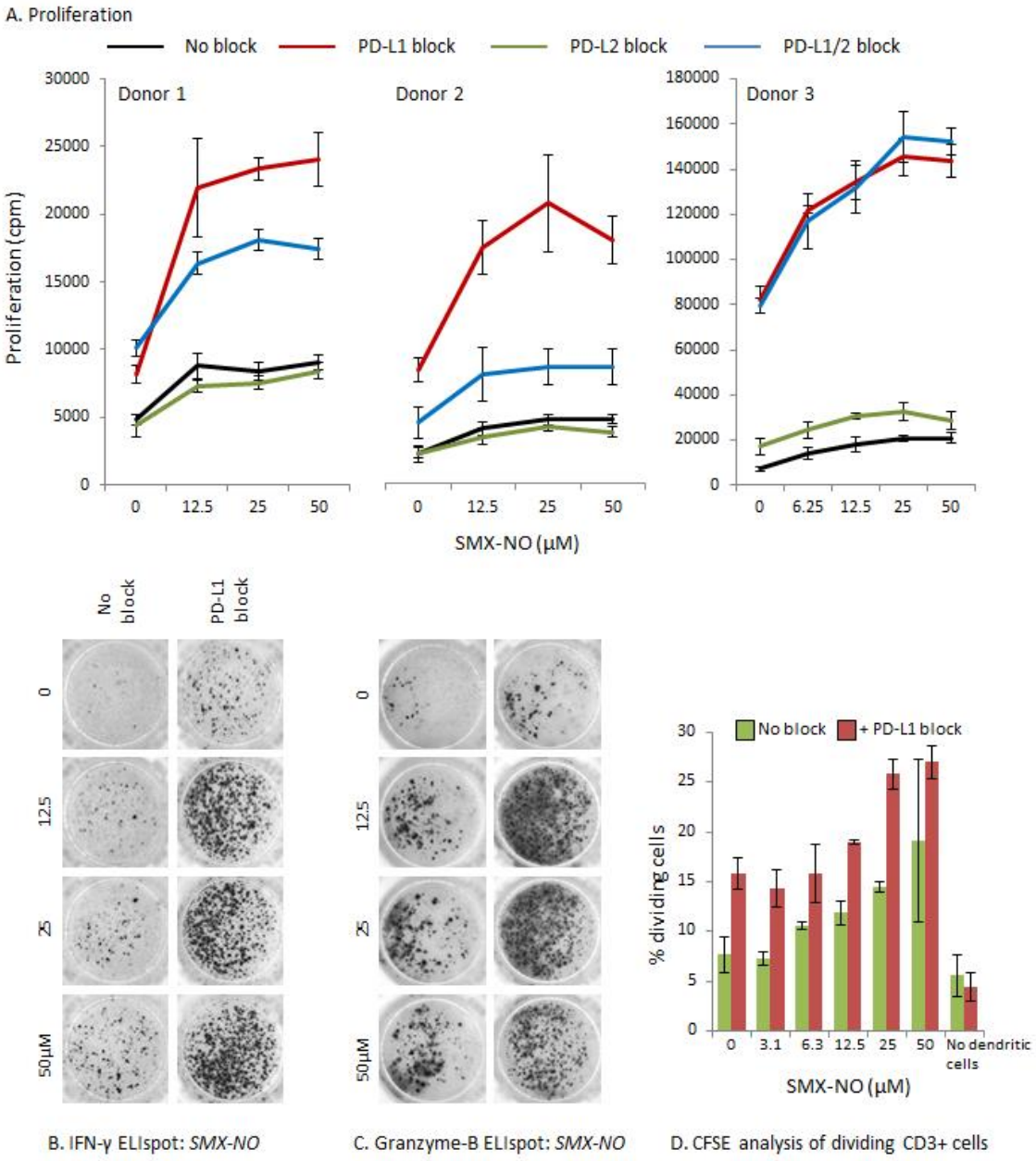
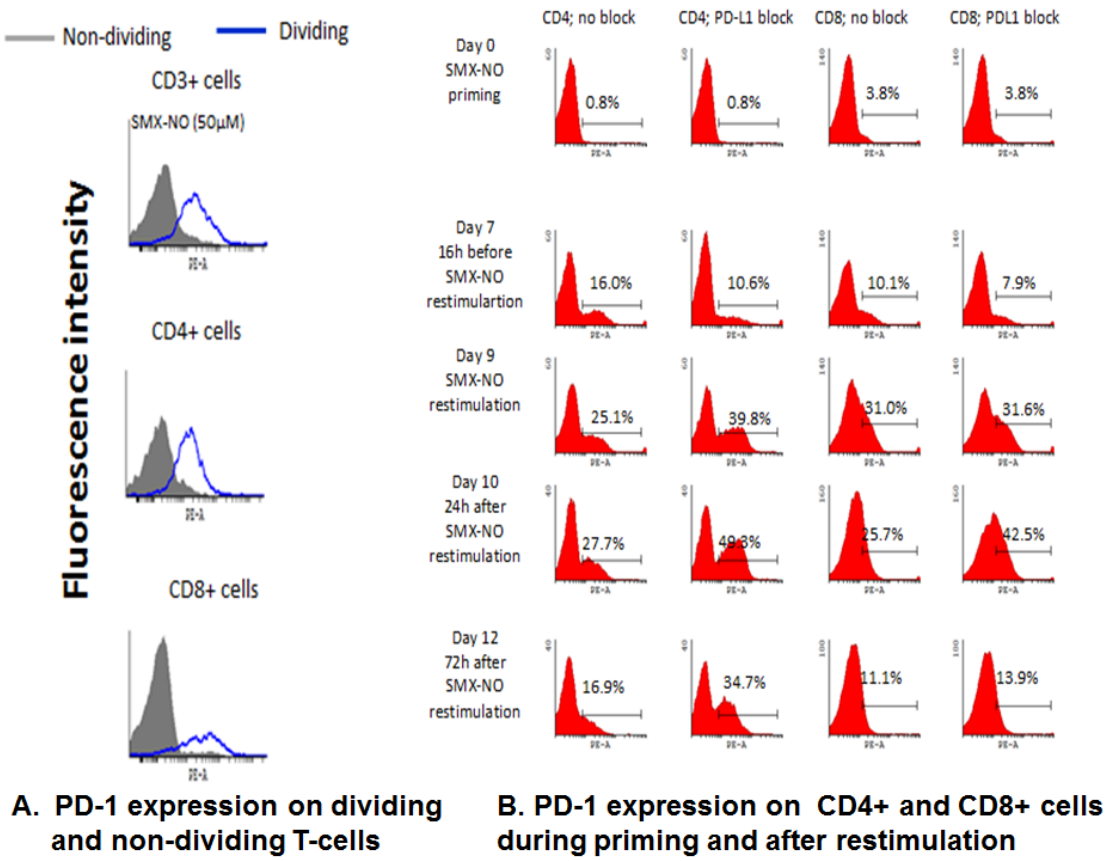


Figure 5.2-SMX.NO-specific priming of naïve T-cells with and without PD-L1/PD-L2 block. **(A)** Antigen-specific T-cell responses to SMX.NO measured by [^{3}H]-thymidine incorporation. Naïve CD3+ T-cells were co-cultured with SMX.NO and dendritic cells at a ratio of 25:1 in the presence or absence of PD-L1/PD-L2 block for 8 days. The cultures were plated and restimulated with fresh dendritic cells and SMX.NO in 96-well U-bottom plates in a final volume of 200 µl. Cells were cultured for 3 days and [^{3}H]- thymidine (0.5 µCi) was added for the final 16 hours incubation. The data show mean SD of triplicate wells. **(B, C)** Antigen-specific T-cell responses measured by IFN-γ and granzyme-B ELISpot, culture conditions were the same as above. Cells were incubated for 48 hours and spots developed according to the manufacturer's instructions. **(D)** Antigen-specific T-cell responses measured by CFSE content. T-cells were labelled with CFSE prior to incubation for 72 hours. Cells were analysed by flow cytometry (a minimum of 50,000 cells were counted).

Analysis of CFSE staining by flow cytometry allows a more in depth analysis of proliferation by distinguishing between $CD4^+$ and $CD8^+$ T-cell populations (figure 5.2D). SMX.NO stimulated the activation of naïve $CD4^+$ and $CD8^+$ T-cells in a concentration-dependent fashion; similar to the [^{3}H]-thymidine data, 25-50 μM SMX.NO were the optimal stimulatory drug concentrations. Interestingly, PD-L1 block did not increase the number of $CD3^+$, $CD4^+$ or $CD8^+$ cells that were stimulated to divide in the presence of SMX.NO (figure 5.3A).

5.6.2 PD-1 expression is enhanced on drug-primed dividing T-cells

CFSE staining was used to measure PD-1 on dividing and non-dividing T-cells. In initial experiments, PD-1 expression was found to be significantly upregulated on dividing $CD3^+$, $CD4^+$ and $CD8^+$ cells 48 hours after SMX.NO restimulation (figure 5.3A). In subsequent experiments, PD-1 expression was measured during SMX.NO priming and for 72 hours after restimulation. PD-1 was not detectable on naïve $CD4^+$ and $CD8^+$ cells. After priming, a small population of PD-1 positive cells was seen on day 7, both in the presence and absence of PD-L1 block. After restimulation with SMX.NO (day 9), PD-1 expression was rapidly upregulated on 20-40% of $CD4^+$ and $CD8^+$ cells in a transient fashion (figure 5.3B; columns 1 and 3). PD-1 reverted back to pre-restimulation levels within 48-72 hours. In the presence of PD-L1 block, the increase in PD-1 expression was sustained (figure 5.3B; columns 2 and 4). Greater than 35% of CD4+ cells stained positive for PD-1 72 hours after restimulation.



A. PD-1 expression on dividing and non-dividing T-cells

B. PD-1 expression on CD4+ and CD8+ cells during priming and after restimulation

Figure 5.3-PD-1 expressions on CD3⁺, CD4⁺ and CD8⁺ T-cells. (A) PD-1 expression on dividing and non-dividing cells. Naïve CD3⁺ cells were co-cultured with SMX.NO and dendritic cells at a ratio of 25:1 in the presence or absence SMX.NO for 8 days. The cultures were labelled with CFSE and restimulated with fresh dendritic cells and SMX.NO for 3 days. Cells were labelled with an anti-PD-1 antibody and analysed by flow cytometry. A minimum of 50000 cells were acquired using FSC/SSC characteristics and CD3⁺, CD4⁺ or CD8⁺ cells were gated for analysis. (B) Naïve CD3⁺ cells were labelled with CFSE and co-cultured with SMX.NO and dendritic cells in the presence and absence of PD-L1 block for 8 days. The cultures were plated and restimulated with fresh dendritic cells and SMX.NO in 96-well U-bottom microplates in a final volume of 200 µl. An aliquot of cells were taken throughout the culture period and PD-1 expression measured by flow cytometry. A minimum of 50000 cells were acquired using FSC/SSC characteristics and CD4⁺ or CD8⁺ cells were gated for analysis.

5.6.3 Drug-primed T-cells secrete IL-22, but not IL-17

Cutaneous reactions to drugs have been classified previously according to the phenotype and function of antigen-specific T-cells. The discovery of new T-cell subsets (e.g., Th17, Th22 cells) may render this classification somewhat obsolete; however, it should be noted that, a role for IL-17 and IL-22 in drug-specific reactions has yet to be defined. To explore whether drug-responsive T-cells produce IL-17 and/or IL-22, detailed cytokine signatures were studied following SMX.NO priming under different polarizing conditions.

In initial priming experiments, naïve $CD3^+$ T-cells were cultured with dendritic cells and SMX.NO in the absence of polarizing cytokines. These cells were then harvested, restimulated with SMX.NO and assayed for IFN-γ, IL-13, IL-17 and IL-22 secretion. SMX.NO-specific secretion of IFN-γ, IL-13 and IL-22 was observed. However, IL-17 release was not detected (Figure 5.4).

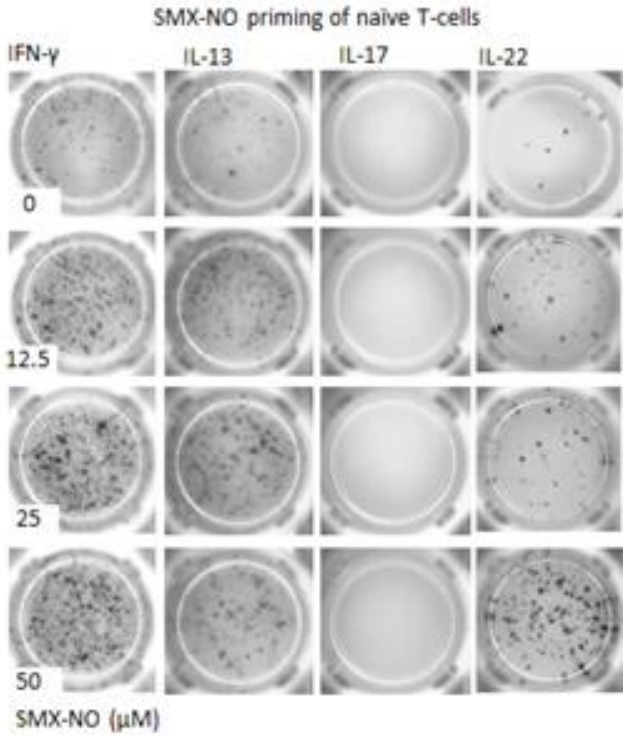


Figure 5.4-IL-22 secretion by T-cells exposed to SMX.NO. Naïve $CD3^+$ T-cells were co-cultured with SMX.NO and dendritic cells for 8 days. The cultures were plated and restimulated with fresh dendritic cells and SMX.NO. Antigen-specific T-cell responses were measured by IFN-γ, IL-13, IL-17 and IL-22 ELIspot. Figure shows representative data from 1 out of 3 experiments.

5.6.4 Generation of T-cell clones and characterization of cytokine secretion profiles

To characterize the functionality of drug-responsive T-cells and the way in which PD-1 signalling influences effector T-cell responses, SMX.NO specific T-cell clones isolated following *in vitro* priming and from hypersensitive patient PBMCs were studied.

Two hundred and eighty three $CD4^+$ T-cell clones were generated following SMX.NO priming of naïve cells. Nineteen were found to proliferate in the presence of SMX.NO (no drug, 1551±410cpm; SMX.NO, 4880±913cpm). The number of clones generated from hypersensitive patients, their CD phenotype and the SMX.NO-specific proliferative response are summarized in Table 5.1.

Proliferation of representative patients and volunteer TCCs is illustrated in figure 5.5. CD4 and CD8 characterization was performed to determine the cell surface phenotype of drug specific TCCs. All the SMX.NO-specific T-cell clones were found to be $CD4^+$ (figure 5.6).

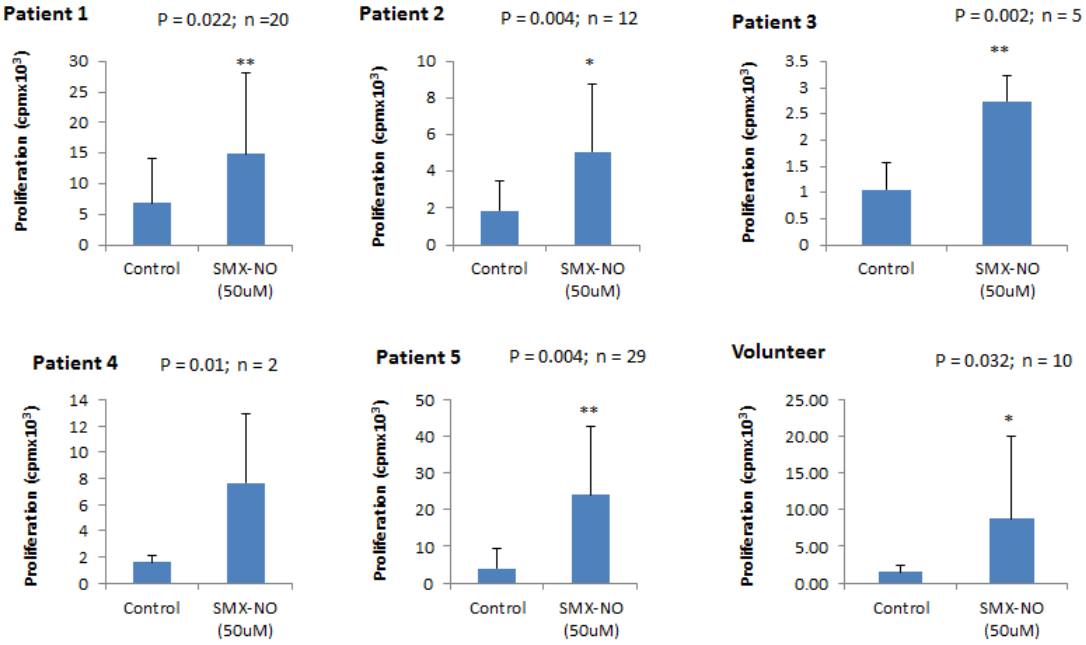


Figure 5.5-Antigen-specific T-cell proliferation. SMX.NO-specific T-cell clones generated from five SMX-hypersensitive patients and one volunteer (5×10^4 cells, 50 µL) was co-incubated with autologous EBV-transformed B-cell line (1×10^4) in the presence and absence of SMX.NO (50 uM) and incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 48 hours. T-cell proliferation was determined by [^{3}H]-thymidine incorporation. Data represent mean of duplicate wells.

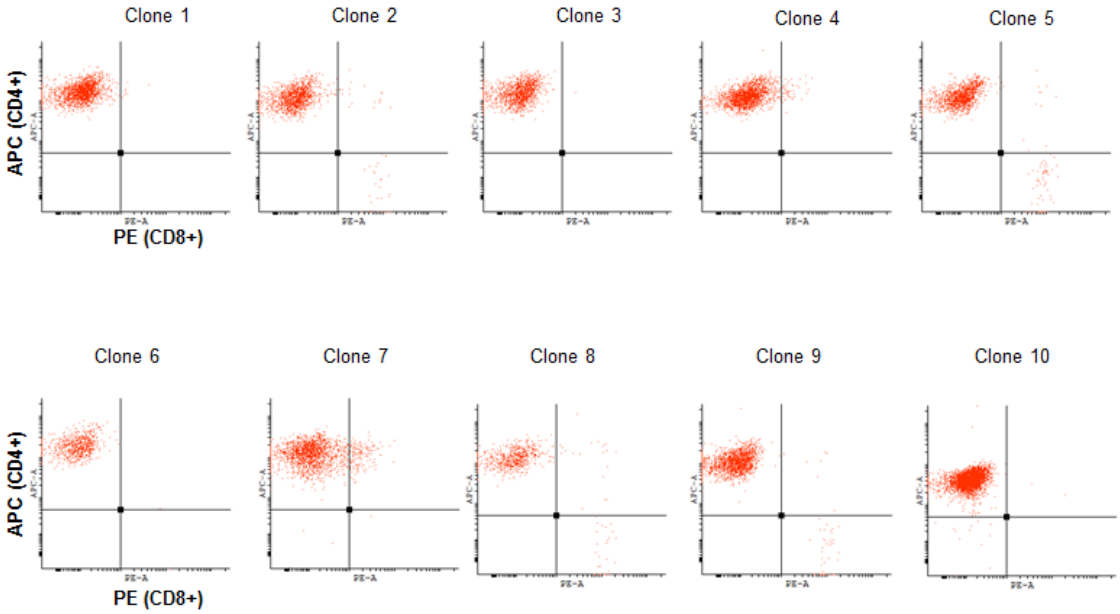


Figure 5.6-CD4⁺/CD8⁺ phenotyping of 10 selected SMX.NO specific T-cell clones. T cell clones were labelled with anti-CD4⁺ and anti-CD8⁺ antibodies and analysed by flow cytometry using a FACS Canto II. A minimum of 50,000 lymphocytes were acquired using FCS/SSC characteristics.

SMX.NO-responsive clones generated from the SMX-naïve volunteer secreted large quantities of IFN-γ, IL-5 and IL-13 following activation (Figures 5.8). Approximately 50% of clones also secreted IL-22. The detection of IL-22 secreting clones was not dependent on priming under Th22 polarizing conditions as clones were not maintained in the presence of Th22 polarizing cytokines. No IL-17 secreting SMX.NO-specific TCCs were detected.

Clones from hypersensitive patients were found to proliferate and secrete IFN-γ, IL-5 and IL-13 following SMX.NO stimulation. Analogous to the *in vitro* priming studies, around 50% of clones also secreted IL-22. IL-17 secretion was only detected with 1 clone (Figures 5.7 and 5.8).

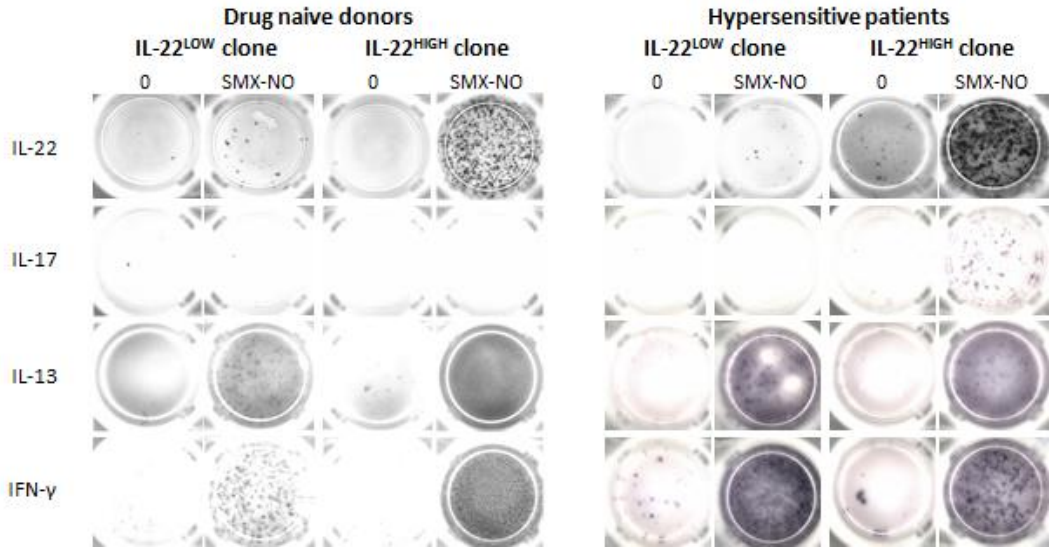


Figure 5.7-Cytokine secretion by SMX.NO responsive CD4⁺ clones. Cytokine profile of representative SMX.NO-specific IL-22^{high}- and IL-22 ^{low}-secreting clones generated from drug-naïve donor after *in vitro* priming and hypersensitive patients.

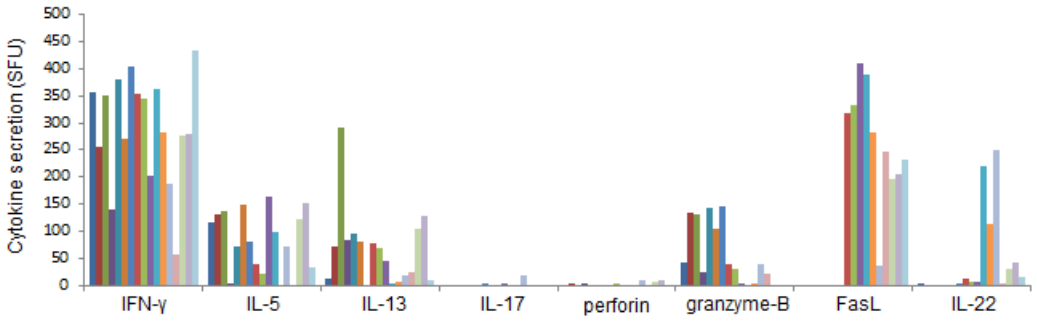


Figure 5.8-Cytokine secretion by SMX.NO-responsive CD4⁺ clones. Analysis of SMX.NO-specific cytokine and cytolytic molecule secretion from seventeen CD4⁺ clones by ELISpot. T-cell clones (5×10^4 cells, 50 µL) were co-incubated with autologous EBV-transformed B-cell line (1×10^4 cells, 50 µL) in the presence and absence of SMX.NO (50 uM) for 48 hours and spots developed according to the manufacturer's instructions.

5.6.5 Skin homing receptor expression and migration of IL-22 secreting T-cell clones

Seventeen SMX.NO-specific clones with a strong growth pattern were selected to explore in detail the profile of secreted cytokines and cytolytic molecules. As described above, all clones secreted IFN-γ, IL-5 and IL-13 when stimulated with SMX.NO. IL-22 production was detected from approximately 50% of the clones. Interestingly, the clones were also found to secrete either FasL or granzyme B, but not perforin (figure 5.8). The IL-22^{high} clones belonged exclusively to the FasL producing subset (figure 5.9). A panel of six clones was then selected (2 FasL^{high} IL-22^{high}granzyme B^{low}; 1 FasL^{high} IL-22^{low}granzyme B^{low}; 3 FasL^{low} IL-22^{low}granzyme B^{high}) to explore which clones expressed CCR4, CCR10 and CLA and hence have the ability to migrate towards skin. All FasL^{high}IL-22^{high} clones expressed high levels of CCR4, CCR10 and CLA (figure 5.9) and migrated in the presence of CCL17 and CCL27 (figure 5.10).

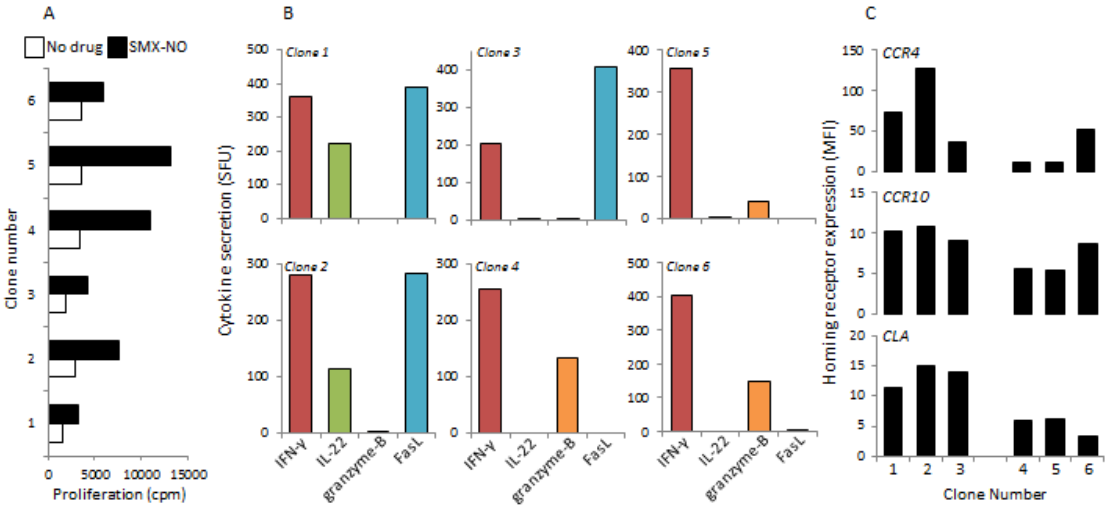


Figure 5.9-Proliferation, cytokine secretion profile and skin homing receptor expression of T-cell clones. (A) Six selected SMX.NO specific T-cell clones ($5x10^4$) were co-incubated with autologous EBV-transformed B-cell lines (1×10^4) with or without SMX.NO (50uM) under an atmosphere of 95% O_2/5% CO_2 for 48 hours. T-cell proliferation was determined by [^{3}H]-thymidine incorporation. Data represent mean of duplicate wells. **(B)** Cytokine and cytotoxic molecules secretion profile (IFN-Y, IL-22, granzyme-B and Fas ligand) for six selected SMX.NO-responsive $CD4^+$ clones was analysed by ELISpot assay. T-cells were incubated for 48 hours and spots developed according to the manufacturer's instructions. **(C)** Flow cytometric analysis of CCR4, CCR10 and CLA expression on SMX.NO responsive clones. Three FasL and/or IL-22 secreting and three granzyme-B secreting clones were selected for the analysis. A minimum of 50,000 lymphocytes were acquired using FCS/SSC characteristics.

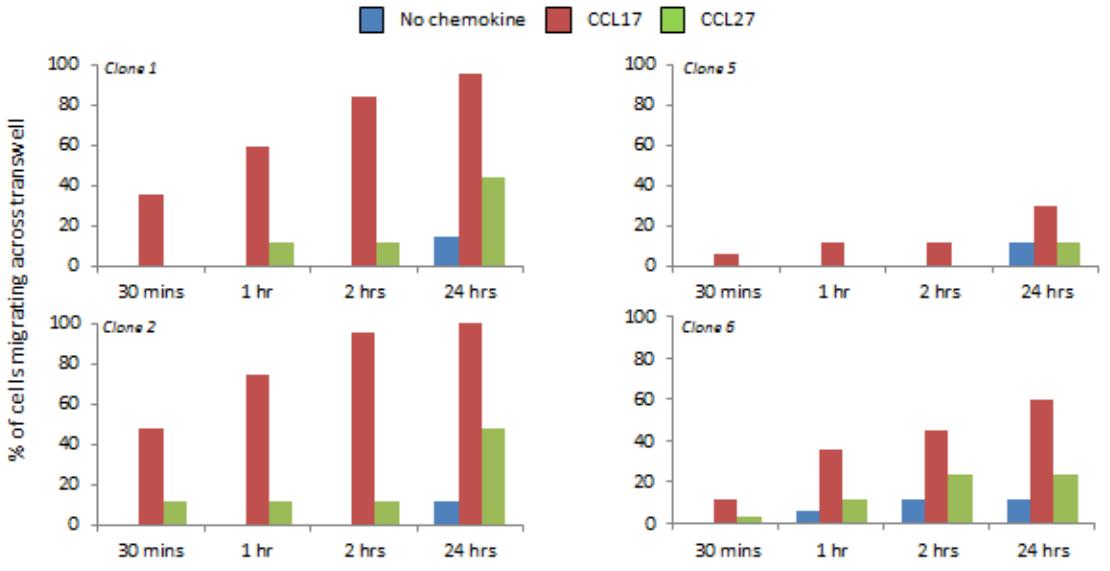


Figure 5.10-Chemotaxis of FasL/IL-22 and granzyme-B secreting clones promoted by CCL17 or CCL27. SMX.NO-specific T-cell clones (10^4) were placed in the top chamber of a transwell and the chemokines in the bottom. The number of cells migrating across the transwell was measured using a haemocytometer after 0.5-24 hours.

5.6.6 PD-1 expression on SMX specific TCCs and PD-L1/2 expression on antigen presenting cells

Although PD-1 is most commonly described as a marker of T-cell exhaustion, it has also been reported that PD-1^{high} cells are highly cytotoxic and/or proliferative (Riley 2009; Zelinskyy et al. 2011). Thus, our SMX.NO-specific clones were used to (1) measure PD-1 expression on individual clones, (2) explore the relationship between PD-1 expression and effector function and (3) analyse whether PD-L1 block alters the levels or profile of cytokines secreted following antigen stimulation.

Flow cytometric analysis of PD-1 on 40 clones revealed a 4 fold difference in expression (figure 5.11A). PD-1 was stably expressed on the surface of clones over a period of 10days ± SMX.NO stimulation (figure 5.11B). PD-1 expression did not correlate with the strength of the drug-specific proliferative response or secretion of IFN-γ, IL-5, IL-13, IL-17, IL-22, perforin, granzyme B or FasL (r^2 less than 0.4 for all parameters tested, r^2 = 0.5 for IFN-γ) as shown in figure 5.12. The 17 clones depicted in figure 5.8 were used for the comparisons.

A number of studies have reported a differential expression of PD-L1 and PD-L2 on various immune cells. We therefore examined the levels of both proteins on dendritic cells and EBV-transformed B-cells. PD-L1 and PD-L2 expression on dendritic cells was 5 fold higher than EBV-transformed B-cells (figure 5.13).

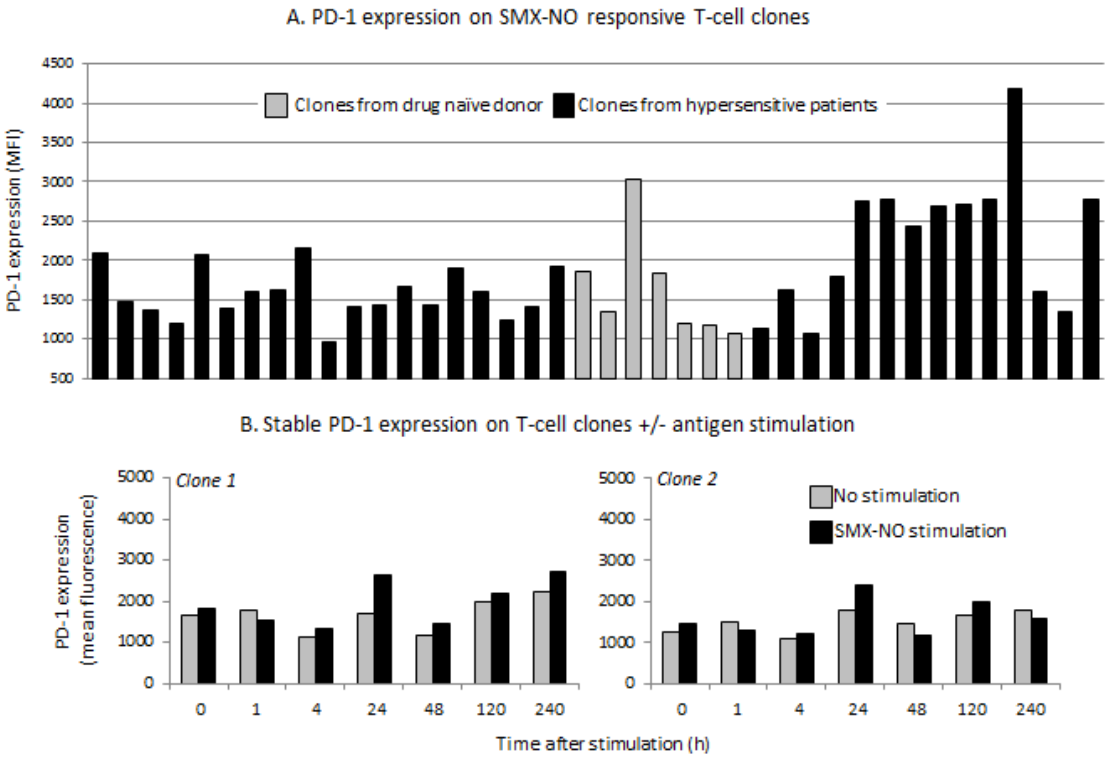


Figure 5.11-PD-1 expression on SMX.NO specific CD4⁺ T-cell clones. (A) PD-1 expression on SMX.NO-specific CD4⁺ clones. Cells were labelled with an anti-PD-1 antibody and analysed by flow cytometry. A minimum of 50,000 cells were acquired using FSC/SSC characteristics. **(B)** PD-1 expression on dividing and non-dividing CD4⁺ clones. Clones were cultured with or without SMX.NO and PD-1 expression was measured for 10 days.

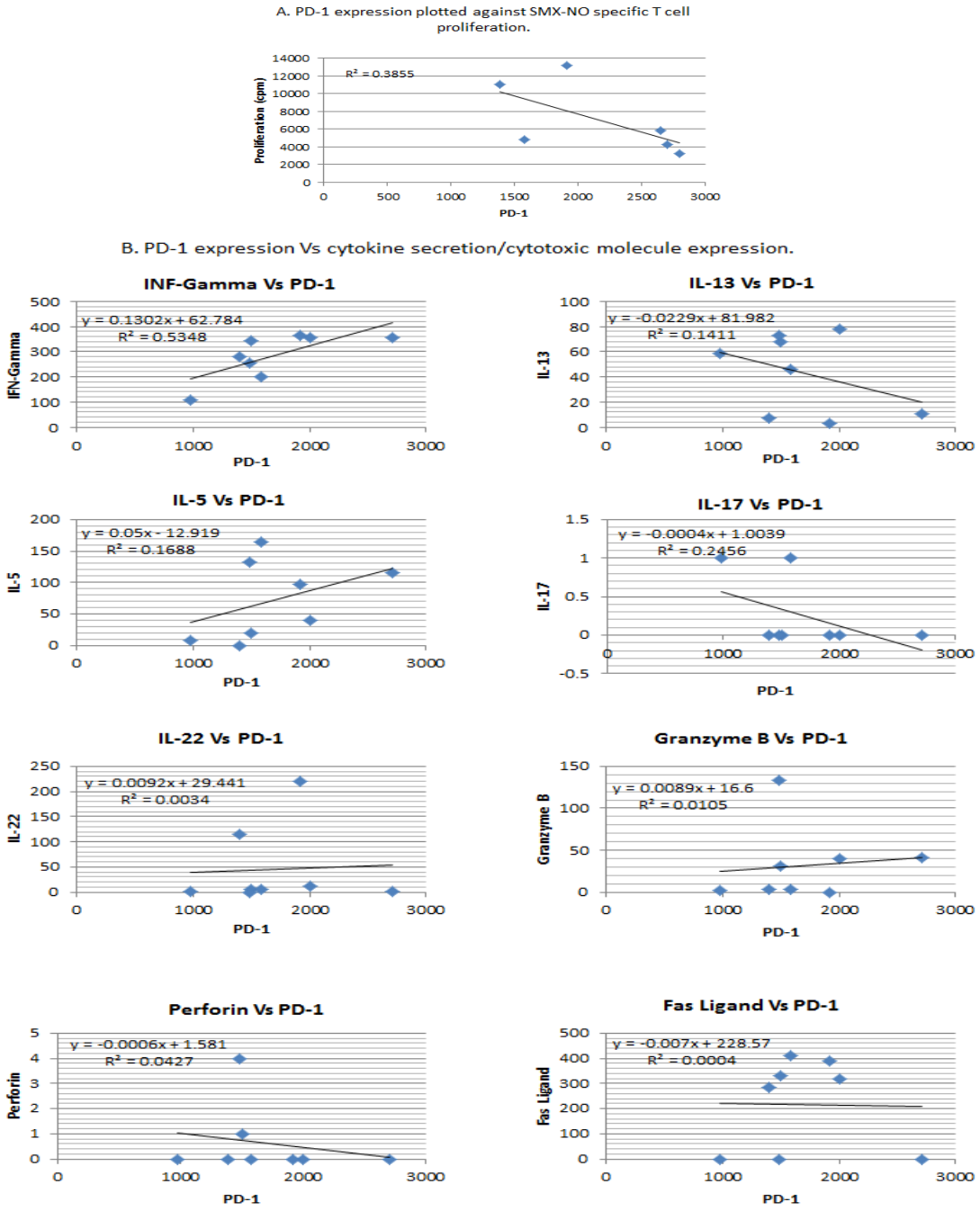


Figure 5.12–(A) Correlation of PD-1 with T-cell proliferation **(B).** PD-1 expression on SMX.NO responsive T-cell clones was plotted against IFN-γ, IL-5, IL-13, IL-17, and IL-22, perforin, granzyme B or FasL. PD-1 was represented as mean fluorescence of 50,000 SMX.NO-specific T-cell clones.

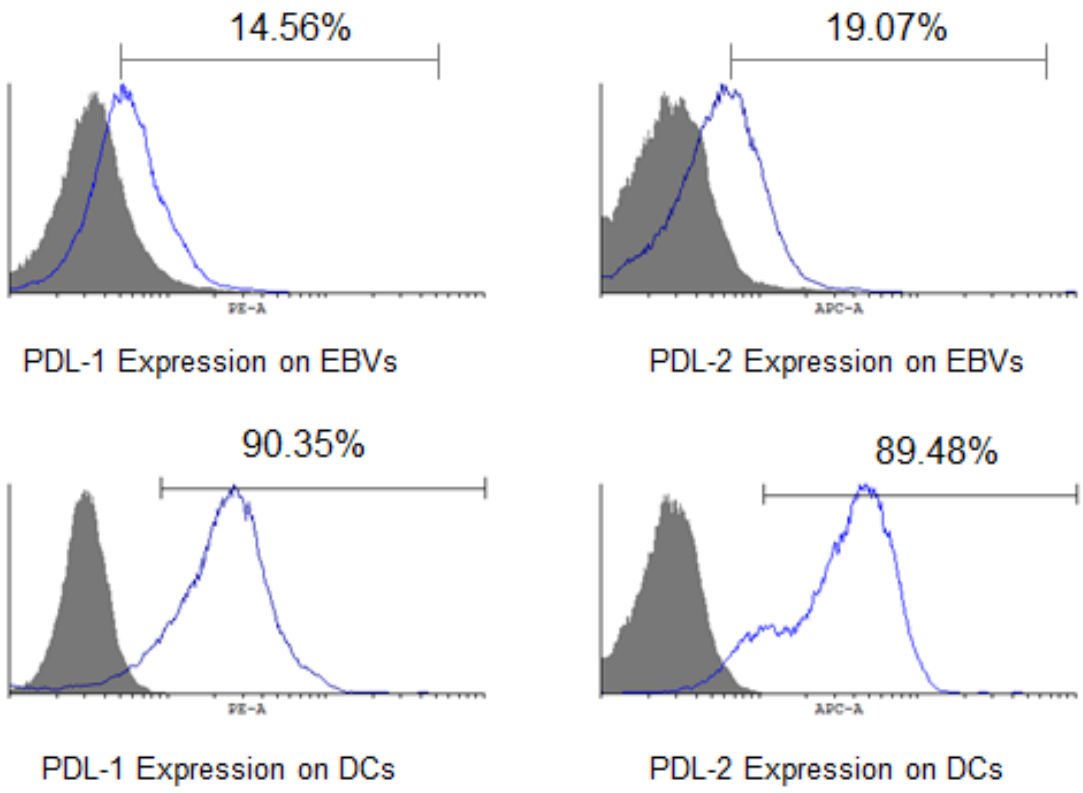


Figure 5.13–PD-L1/PD-L2 expression on APCs. Differential expression of PD-L1 and PD-L2 on EBV transformed B-cells and dendritic cells using anti-PD-L1/PD-L2 antibodies by flow cytometry. PD-L2 was represented as mean fluorescence of 50,000 SMX.NO specific T-cell clones. Grey shades represent baseline auto-florescence while blue lines represent either PDL-1 or PDL-2 expression on EBVs and DCs.

5.6.7 Role of PD-1 block on effector T-cell response

PD-L1 block had no effect on the proliferation of TCCs stimulated with SMX.NO (figure 5.14A). In contrast, PD-L1 block resulted in a modest increase in IFN-γ, IL-13 and granzyme-B secretion (figure 5.14B and C). Blocking the PD-1/PD-L2 signalling pathway had no effect on either T cell proliferation or interferon gamma secretion following SMX.NO stimulation (figure 5.15).

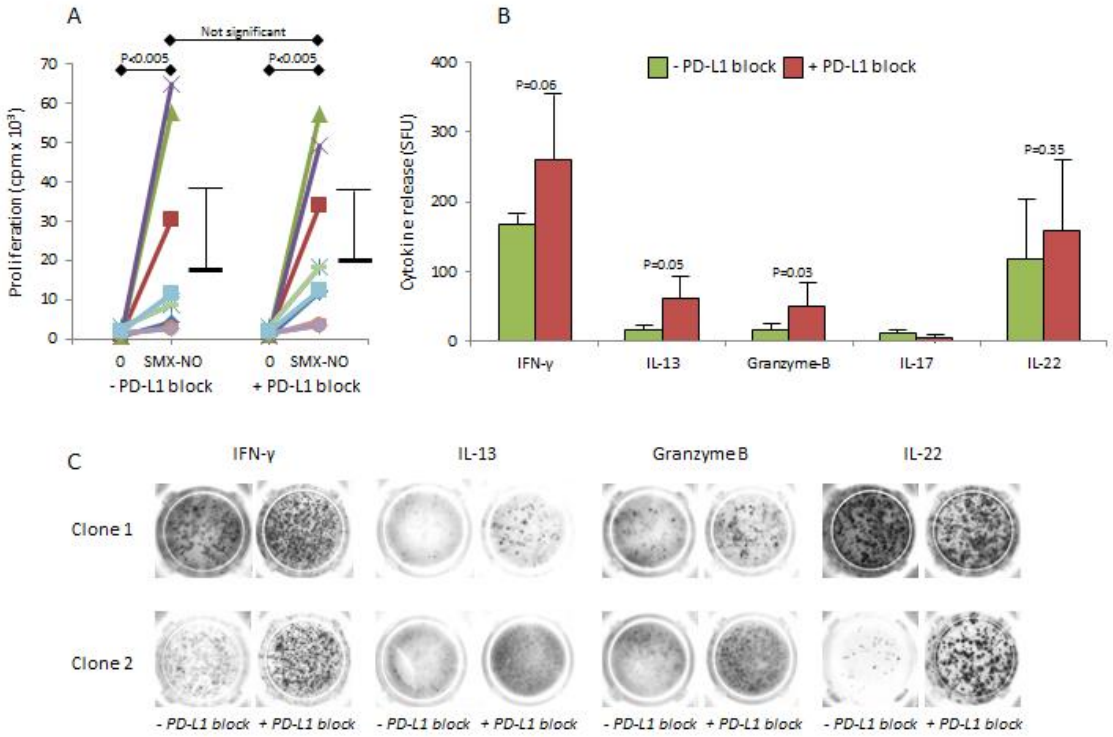


Figure 5.14–(A) Proliferative responses and **(B)** cytokine secretion from SMX.NO-specific T-cell clones with and without PD-L1 block. Ten $CD4^+$ clones were cultured with SMX.NO in the presence or absence of PD-L1 block for 3 days. Responses were measured by [^{3}H]-thymidine incorporation and ELISpot. **(C)** ELISpot images show differences in cytokine secretion from two representative clones ± PD-L1 block.

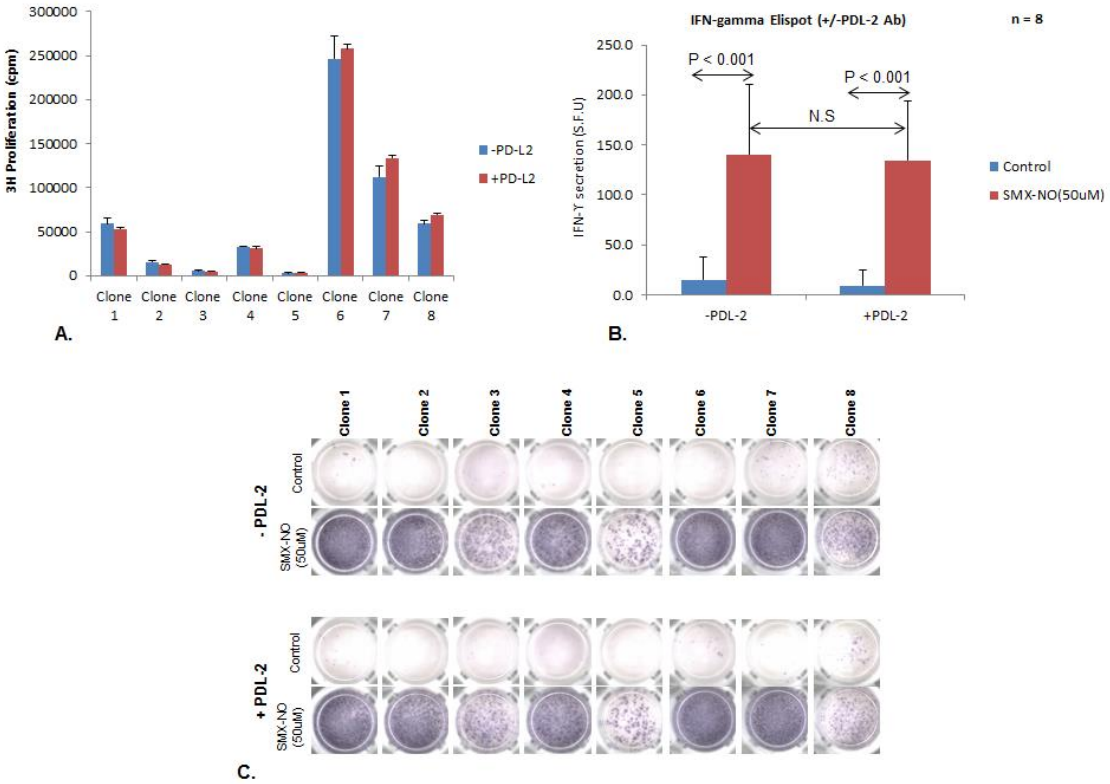


Figure 5.15-Proliferative responses and IFN-Ɣ secretion from SMX.NO-specific clones with and without PD-L2 block. Eight $CD4^+$ clones were cultured with SMX.NO (50 μM) in the presence or absence of PD-L2 block for 48 hours (**(A and B)**. T-cell activation was measured by [^{3}H]-thymidine incorporation and IFN-Ɣ ELISpot. **(C)** ELISpot images show no significant differences in INF-Ɣ secretion for eight representative clones ± PD-L2 block.

5.7 Discussion

In the present study we have focused on regulation of drug antigen-specific T-cell priming through the PD-1/PD-L1 pathway, the way in which PD-1 signalling influences effector T-cell responses and the functionality of drug-responsive clones generated through *in vitro* priming or those isolated from hypersensitive patient PBMCs. The inhibitory function of PD-1 relies on the presence of an immunoreceptor tyrosine-based switch motif. On activation, the switch becomes phosphorylated and subsequently recruits the protein tyrosine phosphatase SHP-2. This causes the inhibition of downstream pathways through the dephosphorylation of proteins such as CD3 and ZAP70 (Okazaki and Honjo 2006; Riley 2009; Zelinskyy et al. 2011) preventing further T-cell stimulation.

To assess the effect of PD-ligand blockade and in particular whether this could be used as an immunogenic boost to enhance drug-specific stimulation of naïve T-cells we utilized an *in vitro* T-cell priming assay and the model drug hapten SMX.NO. In agreement with our previous study, an eight day culture period was sufficient to activate naïve $CD3^+$ T-cells and SMX.NO-specific responses were readily detectable following antigen recall using readouts for proliferation and IFN-γ or granzyme-B secretion. CFSE staining revealed that naïve $CD4^+$ and $CD8^+$ T-cells were activated during priming. The dividing cells were $CD45RO^+$, indicating a change in phenotype from naïve to memory. PD-1 expression was induced on dividing T-cells during priming and following antigen recall. An increase in the magnitude of the drug-specific proliferative response and levels of IFN-γ/granzyme-B secretion was seen when naïve T-cells were exposed to PD-L1-block. In contrast, PD-L2 block had no effect on the activation of naïve T-

cells. It is not clear why PD-L2 block did not enhance the priming of naïve T-cells against SMX.NO. One potential explanation is that B7.1 (CD80), a CD28 co-stimulatory ligand, is known to interact with PD-L1, but not PD-L2 (Butte et al. 2007), this however requires further investigation.

Interestingly, PD-L1 block did not increase the number of SMX.NO-specific T-cells generated during priming. Hence, PD-1/PD-L1 signalling decreases the strength of the antigen-specific proliferative response and cytokine release, but does not regulate the number of T-cells primed against drug-derived antigens. These data are in agreement with previous studies showing that PD-L1 block results in a reversal of T-cell anergy and enhanced T-cell responses against peptide antigens (Chikuma et al. 2003).

Whether PD-1 signalling regulates the activation of antigen-specific memory T-cell responses has yet to be fully defined. Previous studies show that PD-1^{high} cells can be highly cytotoxic and that PD-1 expression might be a marker of effector memory function, which seems counterintuitive (Duraiswamy et al. 2011; Zelinskyy et al. 2011). Thus, using SMX.NO-responsive clones generated from healthy donors through priming and from hypersensitive patients, we assessed whether PD-1 expression correlated with the strength of the antigen-specific proliferative response and/or secretion of cytokines/cytolytic molecules. Furthermore, PD-L1/2 blocking antibodies were used to assess whether PD-1 signalling regulates the activation of antigen-specific memory T-cells. Detailed analysis of 40 clones revealed (1) a four-fold variation in PD-1 expression on T-cells, (2) PD-1 was stably expressed for up to 10 days after antigen stimulation, and (3) there was no correlation between PD-1 expression and the magnitude of the drug-specific proliferative response or secretion of

cytokines. Nevertheless, subtle increases in IFN-γ, IL-13 and granzyme-B secretion were observed when clones were stimulated with SMX.NO in the presence of PD-L1 block. PD-L2 block had no significant effect on either the drug-specific proliferation of TCCs or IFN-ϒ secretion profile.

Immunological drug reactions cause a variety of different skin conditions that can be characterized in terms of the phenotype and function of antigen-specific T-cells. Histological analysis of inflamed skin from patients with maculopapular eruptions, which accounts for the majority of SMX reactions, reveals the presence of cytotoxic $CD4^+$ T-cells that secrete granzyme-B. Furthermore, drug-specific T-cells isolated from patient blood are mainly $CD4^+$ and secrete a mixed panel of Th1/Th2 cytokines including IFN-γ, IL-5 and IL-13 (Pichler et al. 2002; Pichler 2003; Elsheikh et al. 2011). However, the discovery of new T-cell populations (e.g., Th9, Th17, and Th22) renders this classification somewhat obsolete. For this reason, we characterized the functionality of SMX.NO-specific T-cells generated through *in vitro* priming and from SMX-hypersensitive patients. Following antigen recall, the SMX.NO primed T-cells from healthy donors were found to secrete IFN-γ, IL-13 and IL-22, but IL-17 secretion was not detected. $CD4^+$ clones isolated from the priming assay also secreted IFN-γ, IL-5 and IL-13, but no IL-17. IL-22 secretion was detected from approximately 50% of the clones. A similar pattern of cytokine secretion was seen with $CD4^+$ clones (IFN-γ^{high} IL-5^{high} IL-13^{high} IL-22^{low} and IFN-γ^{high} IL-5^{high} IL-13^{high} IL-22^{high}) isolated from SMX-hypersensitive patients. IL-22 is a cytokine that modulates tissue responses as expression of the IL-22R1 receptor is restricted to non-haematopoietic cells. In skin, the IL-22 receptor is expressed at high levels on keratinocytes and IL-22 has been found to enhance keratinocyte

proliferation and inhibit terminal differentiation (Boniface et al. 2005). Furthermore, IL-22 has been shown to mediate inflammatory responses in patients with psoriasis and IL-22 secreting cells have been identified in patients with allergic contact dermatitis (Eyerich et al. 2010; Akdis et al. 2012; Cavani et al. 2012). Our data is, however, the first to show production of IL-22 alongside IFN-γ by antigen-specific T-cells from drug-hypersensitive patients.

Given the heterogeneous secretion of IL-22 by individual clones, the release of cytolytic molecules (perforin, granzyme-B and FasL) and expression of skin-homing chemokine receptors were also measured using ELISpot and flow cytometry, respectively. These studies identified two subsets of drug-specific TCCs classified according to the production of either granzyme B *or* FasL. Importantly, the IL-22 secreting clones produced FasL following antigen stimulation. They expressed high levels of the skin homing receptors CCR4, CCR10 and CLA and migrated towards CCL17 and CCL27, indicating that the receptor expression was functionally relevant. Collectively, these studies identify two pathways of killing by drug-specific TCCs. The FasL and IL-22 secreting clones may be crucial mediators of the immunological reaction as they are programmed to migrate towards skin.

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is another negative regulator of effector T- cell function that has been extensively researched (Walunas et al. 1994; Pentcheva-Hoang et al. 2009; Yokosuka et al. 2012). CTLA-4 binds to its ligands; B7-1 (CD80) and B7-2 (CD85) to inhibit early T-cell activation by preventing IL-2 synthesis, cell cycle development and T-cell receptor signalling (Luhder et al. 2000; Chikuma et al. 2003; Fife et al. 2009). The negative

inhibition by CTLA-4 has been documented to be significantly lower than that induced by PD-1 interaction with its ligands (Parry et al. 2005). Hence, ongoing/future work will examine the role of CTLA-4 signalling in the activation of SMX/SMX.NO-specific T-cells.

In conclusion, our study found that PD-L1/PD-1 signalling negatively regulates the priming of drug antigen-specific T-cells that secrete a heterogeneous pattern of cytokines. Thus, differential PD-L1/PD-1 expression and activity might represent one factor that impacts upon the immune response of human exposed to allergenic drugs.

Chapter 6: Immunogenicity of nitroso-sulphamethoxazole-modified MPO-derived peptides

Contents

6.1 Introduction

Sulphonamides are implicated in a number of cutaneous idiosyncratic adverse drug reactions (Naisbitt 2004; Nassif et al. 2004; Kocak et al. 2006; Kouklakis et al. 2007). SMX is used in combination with trimethoprim in a 5:1 ratio for the treatment of a number of opportunistic infections in HIV patients (Absar et al. 1994; Sibanda et al. 2011) and respiratory tract infections in patients with cystic fibrosis (Hutabarat et al. 1994; Lavergne et al. 2010; Elsheikh et al. 2011). The use of SMX in these patient populations is associated with ADRs in 10-30% of individuals (Pirmohamed and Park 2001; Farrell et al. 2003; Elsheikh et al. 2011). Altered metabolism profiles of SMX and a hyper-reactive immune system in these patients have been put forward as potential reasons for the susceptibility to hypersensitivity to SMX (Carr et al. 1993; Farrell et al. 2003; Lavergne et al. 2006; Elsheikh et al. 2011). Furthermore, trimethoprim undergoes metabolic activation in humans and therefore might also be responsible for some adverse drug reactions in patients taking SMX-trimethoprim combinations (van Haandel et al. 2014).

Hepatic oxidative metabolism of SMX by CYP2C8 and CYP2C9 (Cribb et al. 1995; Wen et al. 2002; Sanderson et al. 2007) and oxidation by myeloperoxidase (MPO) (Cribb et al. 1990) have been extensively characterised and are discussed in chapters 1 and 2. The oxidation of SMX by MPO unlike the oxidation of SMX by P45O cannot be described as a hepatic biotransformation because MPO is not known to be expressed in hepatocytes (Amanzada et al. 2011). Furthermore, the MPO that is reportedly expressed in human Kupffer cells is not known to contribute to the oxidation of SMX in either normal or damaged liver (Brown et al. 2001). Interestingly, Lai WG et al. claimed that 7-

hydroxyfluperlapine, a pre-reactive metabolite of fluperlapine, but not fluperlapine itself, is oxidized by human MPO and covalently modifies the protein *in vitro*(Lai et al. 2000). In acutely injured human liver, MPO was only detected in freshly recruited neutrophil granulocytes (Amanzada et al. 2011). Therefore the oxidation of SMX by constitutive and/or acquired MPO in liver is theoretically possible but remains hypothetical.

MPO-expressing cells that have been utilised in drug metabolism studies include activated neutrophils and monocytes (Daugherty et al. 1994). MPO is also expressed by human Kupffer cells (Brown et al. 2001), the predominant resident macrophages of the liver, and by circulating neutrophils and neutrophils that infiltrate injured tissue.

Hydroxylation of SMX at the N_4 position generates SMX hydroxylamine, SMX.NHOH (Cribb et al. 1995). Auto oxidation of SMX.NHOH results in the formation of nitroso-SMX, SMX.NO (Cribb et al. 1991). SMX.NO is highly reactive and undergoes further reactions to form nitro-SMX and azoxy and azo dimers (Naisbitt et al. 2002). Oxidation of azo compounds produces the azoxy derivatives while dimer formation results from condensation. SMX.NO also undergoes reduction in the presence of excess glutathione or ascorbate to form SMX.NHOH (Lavergne et al. 2006). Immunoblotting and RT-PCR data for enzyme expression in immune cells suggests the expression of low levels of myeloperoxidase in EBV-transformed B-cells used as APCs (chapter 2).

Schnyder et al. (2000) employed the SMX pulsing experiments to investigate the metabolic ability of APCs. This assay involves incubating APCs with either SMX or SMX.NO (control) for 2-8 h followed by extensive washes to remove unbound drug and ^{3}H-thymidine proliferation assay in the presence of drug-specific T-

cells (Schnyder et al. 2000). The authors reported that APCs pulsed with SMX.NO resulted in the modification of APC protein and induced T-cell proliferation. However, SMX-pulsed APCs did not induce T-cell proliferation. In contrast, Elsheikh et al. (2010) reported activation of drug-specific T-cells when APCs were pulsed with SMX for 16 h. Hence, APCs have the ability to metabolise SMX to its protein reactive metabolite (SMX.NO). The details of intracellular protein processing and presentation have been extensively discussed in chapter 1.

Most proteins possess important structural, signalling and/or enzymatic activities. Post-translational modifications of proteins or the absence of such modifications in some situations have been implicated in a number of diseases (Gong et al. 2005; Li et al. 2010). Important post-translational modifications essential for cell signalling and enzymatic activity include: phosphorylation (Hunter 1995), acetylation (Farazi et al. 2001), methylation (Wood and Shilatifard 2004), glycosylation (Spiro 2002) and sulphation (Hemmerich et al. 2004). Modifications of proteins and peptides have been used to enhance desired therapeutic effects of some biological products like insulin (Torosantucci et al. 2011; Yang et al. 2011). In contrast, protein modifications by drugs or their reactive metabolites are thought to be a critical step in a number of adverse drug reactions (Naisbitt et al. 1996; Evans et al. 2004; Park et al. 2005; Walgren et al. 2005; Sanderson et al. 2007; Cheng et al. 2008). Multiple modification sites (13 lysine residues) on human serum albumin by β-lactam antibiotics result in several antigenic epitopes and have been extensively

characterised (Jenkins et al. 2009; Meng et al. 2011; Whitaker et al. 2011; El-Ghaiesh et al. 2012; Jenkins et al. 2013).

Upon oxidative metabolism of SMX, SMX.NO is capable of intracellular protein modification (Cribb et al. 1996; Naisbitt et al. 1996; Naisbitt et al. 1999; Reilly et al. 2000; Naisbitt et al. 2001; Naisbitt et al. 2002; Callan et al. 2009; Lavergne et al. 2009; Castrejon et al. 2010; Elsheikh et al. 2010). *In vivo* haptenation of serum protein in both SMX hypersensitive patients and drug naïve volunteers has been reported (Meekins et al. 1994; Gruchalla et al. 1998). Callan et al. (2009) characterised the modification of human GSH *S*-transferase π (GSTP), human serum albumin (HSA) and DS3 (a synthetic oligopeptide incorporating all of the standard amino acid residues of proteins) by SMX.NO. Mass spectrometry analysis of the resulting adducts revealed formation of a sulphinamide conjugate with DS3 and GSTP, representing a 267-amu mass addition. Modification of GSTP occurred on the reactive cysteine at position 47, with mass additions of 267, 283 and 299 consistent with sulphinamide, *N*-hydroxysulphinamide, and *N*-hydroxysulphonamide adducts, respectively, as illustrated in figure 6.1. Interestingly, the HSA modification reported was on Cys34, generating just the *N*-hydroxysulphinamide adduct.

The biological consequences of these modifications have not been fully defined using either an *in vitro* or *in vivo* system. The anatomical/cellular site of protein modification by small molecules is an important determinant of the nature of the immune response that follows (Weltzien et al. 1996). Proteomic analysis of both natural post-translational protein modifications and protein modifications by drugs and/or their reactive metabolites is possible using LC-MS/MS (Mann

and Jensen 2003; Jenkins et al. 2009; El-Ghaiesh et al. 2012; Jenkins et al. 2013; Monshi et al. 2013) and is important in understanding the molecular basis of immune activation by drug-modified proteins.

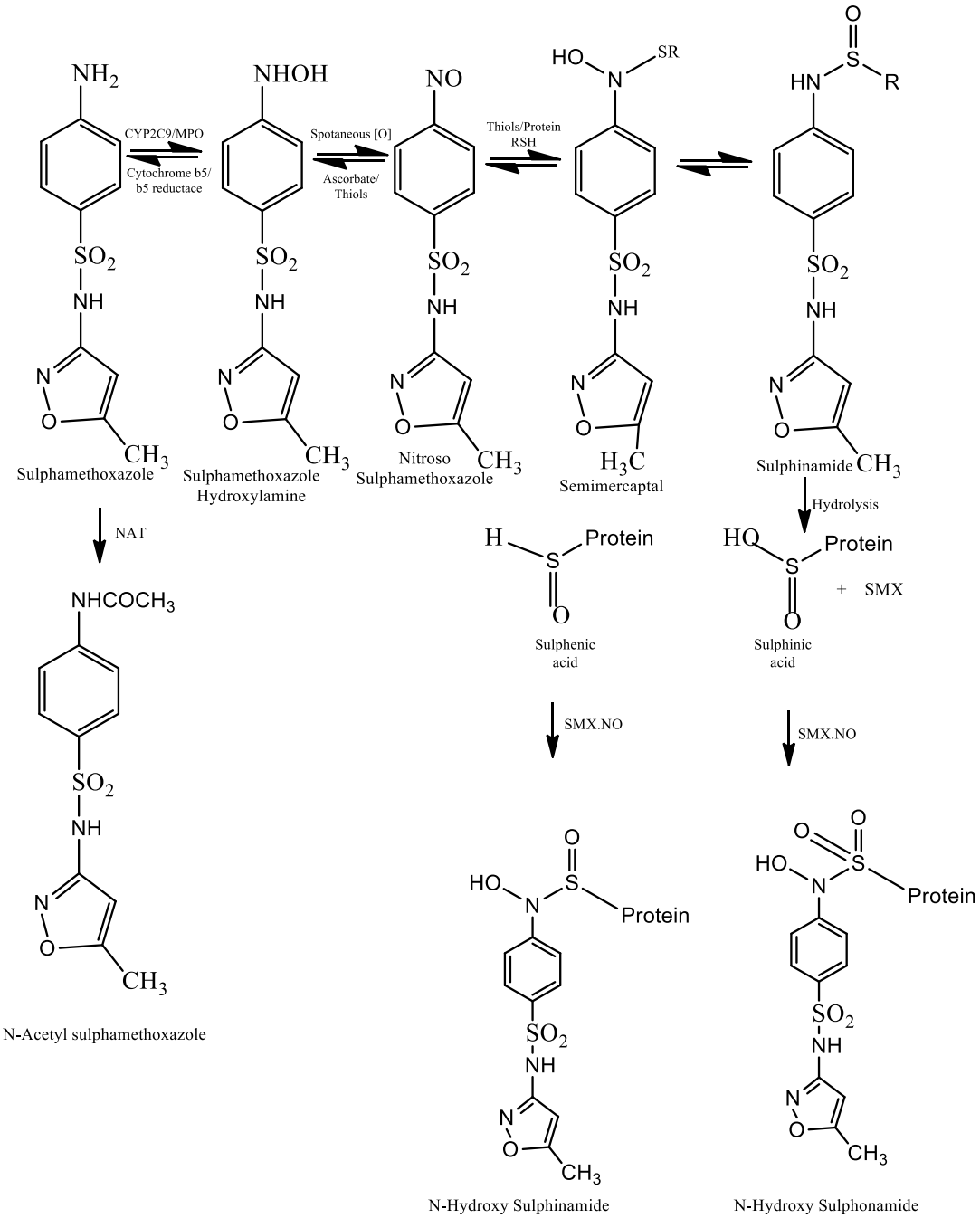


Figure 6.1 Schematic representation of the metabolism and protein haptenation of SMX.

6.2 Aims

Protein haptenation by the SMX.NO reactive metabolite is a determinant in SMX-induced hypersensitivity reactions. Notably, active MPO is present in the skin of patients with drug-induced toxic epidermal necrolysis (Paquet et al. 2010) and a range of immune cells including dendritic cells and B-lymphocytes (Sanderson et al. 2007). Given the intrinsic reactivity of SMX.NO, we hypothesised that modification of cysteine residues would occur in close proximity to the site of metabolite formation. MPO exists as a tetramer (2 long chains + 2 short chains). The protein consists of 15 cysteine residues **(figure 6.2),** made up of four free cysteine residues in each of the dimer subunits (Cys316 in a reduced state). Hence, the objective of this study was to investigate SMX.NO/MPO adduction in an *in vitro* system. The specific aims were

- To characterize the SMX.NO-modified MPO adduct
- To determine the immunogenicity of SMX.NO-modified MPO adduct

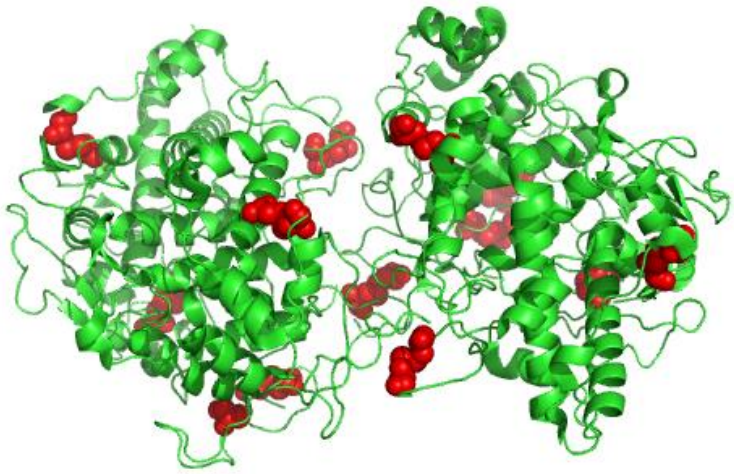


Figure 6.2 Three-dimensional structure of MPO (green ribbon) showing cysteine residues (red spheres).

6.3 Methods

6.3.1 Chemicals and reagents

Human MPO full-length protein was purchased from Abcam (Cambridge, UK). MPO-derived peptides [MPO1 (^{307}ADCIPFFRSCPACPG^{321}), MPO2 (^{307}ADAIPFFRSAPAAPG^{321}) and MPO3 (^{307}ADAIPFFRSCPAAPG^{321})] were synthesised by Invitrogen (Paisley, UK). MPO1 has a sequence that occurs in native MPO; it contained an MHC-II binding sequence within MPO, determined using the immune epitope database (IEDB). MPO2 and 3 were substitution derivatives. All 3 peptides were prepared with a purity >95% as determined by the manufacturer using HPLC. SMX.NHOH was synthesised according to previously published methods (Naisbitt et al. 1996). SMX and peroxidase-conjugated anti-rabbit IgG secondary antibody were obtained from Sigma-Aldrich (Gillingham, Dorset, UK). SMX.NO ($C_{10}H_9N_3O_4S$) was purchased from Dalton Chemical Laboratories Inc. (Toronto, Canada). Anti-SMX antibody was developed by Panigen (Blanchardville, WI, USA). Acetonitrile and methanol were obtained from Fisher Scientific (Leicestershire, UK). Laemmli buffer was bought from Bio-Rad (Hemel Hempstead, Hertfordshire, United Kingdom). Chemiluminescent substrate was purchased from Thermo Scientific (Cramlington, Northumberland, United Kingdom). Amicon Ultra-0.5 mL centrifugal filter units were bought from Merck Millipore Ltd (Carrigtwohill, Ireland).

6.3.2 Isolation of PBMCs and generation of drug-specific T-cell clones

PBMCs were isolated and SMX.NO-specific T-cell clones were generated according to methods described in section 2.3.3.

6.3.3 Generation of EBV-transformed B cells

The EBV-transformed cell line used as APCs was generated as described in section 2.3.5.

6.3.4 Western blotting

Myeloperoxidase (1.5 mg/mL) was incubated in 0.1 M phosphate buffer, pH 7.4, with either SMX (2 mM) or SMX.NO (50 μM) in the presence or absence of hydrogen peroxide (10 μM) or with SMX.NO (50 μM) for between 0-1 hour at 37°C, under an atmosphere of 95% O_2/5% CO_2. A 1:5 molar ratio of MPO: SMX.NO provided optimum binding conditions. MPO incubated in phosphate buffer was utilised as negative control. Samples were processed after 0-60 min for Western blotting. Samples (10 μL) were boiled in reducing Laemmli buffer (2.5 μL) for 10 min and their protein component separated on a 12% SDS-PAGE gel (300 V, 60 mA, and 1 hour) then transferred onto a nitrocellulose membrane (300 V, 250 mA and 1 hour). Non-specific antibody binding sites were blocked using 2.5% reconstituted skimmed milk for 1 hour at room temperature. The blocking was followed by immunoblotting for protein adducts using anti-SMX rabbit antiserum (1:2000 dilutions) overnight at 4°C. PBS-Tween washes were performed at 5-min intervals for a total of 20 min to remove unbound antibody. The nitrocellulose membrane was then incubated with peroxidase-conjugated anti-rabbit IgG antibody (1:5000 dilutions) for 1h at room temperature. The membrane was then developed using chemiluminescent substrate according to the manufacturers' instructions.

6.3.5 Coomassie Blue staining

Coomassie Blue staining of proteins on gels was performed as described in section 2.3.9.

6.3.6 In-gel digests

Bands excised from Coomassie Blue-stained gels were de-stained by adding 100 µL 50% ACN/50 mM ammonium hydrogencarbonate and incubating for 15 min at room temperature with occasional agitation. The supernatants were discarded and the gel bands were dried in a SpeedVac vacuum evaporator (15-20 min). They were rehydrated in 10 mM dithiothreitol/50 mM ammonium hydrogencarbonate (100 µL) and incubated at 56°C for 1 hour. The supernatants were removed and the bands were incubated in 25 µL of a 55 mM iodoacetamide/50 mM ammonium hydrogencarbonate solution for a further 45 min in the dark. The gel pieces were washed with ammonium hydrogencarbonate for 10 min before being dried once more in a SpeedVac. They were then rehydrated in 10 ng/µL trypsin/50 mM ammonium hydrogencarbonate buffer (100 µL) and incubated overnight at 37°C. In order to extract peptides from the gel pieces, they were incubated in a sonicator using 30 µL 60% acetonitrile/1% trifluoroacetic acid for 5 min. After brief centrifugation, the supernatants were collected. This step was repeated once, and then the supernatants were pooled and dried in a SpeedVac. The peptides were resuspended in 0.1% formic acid (10 µL) and 0.5 µL was spotted onto a MALDI target plate or 5 µL was analysed by LC-MS.

6.3.7 In-silico determination of MPO-binding HLA epitopes using the immune epitope database (IEDB screening)

The MPO peptide sequences with the highest probability of associating with MHC II (HLA-DQ) were determined *in silico* using IEDB screening of the MPO amino acid sequence. The IEDB is an internet resource that contains data on antibody and T-cell epitopes for humans, non-human primates, rodents and

other animal species (http://www.iedb.org/). This database contains a variety of resources important for B- and T-cell epitope predictions (Zhang et al. 2008; Kim et al. 2012). Using the MHC II prediction tool on the IEDB, various peptide sequences showing high binding affinities with the HLA-DQ molecule were identified. Our analysis focused on HLA-DQ as previous studies showed HLA-DQ restricted drug presentation using SMX.NO-specific TCCs generated from SMX-hypersensitive patients (chapter 3). Peptides with median inhibitory concentrations (IC_{50}s) of <50 nM were classified as high affinity binders while those with IC_{50S} of <500 nM were classified as intermediate affinity binders. Peptides containing cysteine residues that have free SH groups in the native protein, which therefore might be modified by SMX.NO, were considered for selection. Of particular interest were MPO-derived peptides with cysteine residues at position 309, 316 and 319.

Enhanced SMX.NO haptenation was hypothesized for peptides containing cysteine residues with free SH groups from the long polypeptide chain of MPO (Zeng and Fenna 1992). The long chain of MPO has five intra-chain disulphide bridges and one inter-chain bridge end at Cys153. Evidently, Cys298, Cys309, Cys316, Cys319 have free SH groups.

Since all the SMX.NO-specific TCCs were $CD4^+$ cells, a 15 amino acid peptide, MPO1 (^{307}ADCIPFFRSCPACPG^{321}), with an IC_{50}s of 145 nM, was selected and synthesized along with two similar peptides. All three peptides showed a degree of homology but differed in amino acid residues at positions 309, 316 and 319. LC-MS/MS characterization of SMX.NO-modified peptides was followed by purification. T-cell functional proliferation assays were performed to determine the immunogenicity of SMX.NO-modified MPO peptides. The modified forms of

MPO1 were MPO2 (ADAIPFFRSAPAAPG) and MPO3 (ADAIPFFRSCPAAPG). While MPO2 had alanine substitutions for the cysteine residues at positions 309, 316 and 319, MPO3 had two alanine substitutions at positions 309 and 319.

6.3.8 Generation and purification of SMX.NO-modified MPO peptides

MPO1, MPO2 or MPO3 (1 mg/mL, 100 µL) were incubated with SMX.NO (50 µM, 20 µL) in a 1:5 molar ratio for 1 hour at 37°C, under an atmosphere of 95% O_2/5% CO_2. Removal of unbound SMX.NO and purification of the resulting peptide adduct were carried out using off-line reversed phase (RP) chromatography (described in section 6.3.9).

6.3.9 Reversed phase (RP) HPLC

MPO/MPO derived peptide was incubated with SMX.NO (1:5) at 37°C under an atmosphere of 95% O_2/5% CO_2. The whole incubation was made up to a volume of 500 µL with 0.1% trifluoroacetic acid (TFA); at low pH (~2) as peptides and proteins bind more efficiently to the reversed phase matrix. The sample was then injected onto a Prodigy 150 × 4.6 mm column with 5 µm particle size (Phenomenex) and eluted using a gradient from 95% solvent A (5% acetonitrile/0.1% TFA)/5% solvent B (95% acetonitrile/0.1% TFA) to 50% solvent B in 40 min at a flow rate of 1 mL/min. Fractions of 1 mL were collected and dried in a SpeedVac (Eppendorf) prior to LC-MS analysis.

6.3.10 Generation and purification of SMX.NO-modified recombinant MPO

Recombinant MPO full-length protein (1.5 mg/mL, 100 µL) was incubated with SMX.NO (50 mM, 20 µL) in a 1:5 molar ratio for 1 hour at 37°C, under an

atmosphere of 95% O_2 /5% CO_2. Removal of unbound SMX.NO and the purification of resulting adducts were carried out using either RP chromatography or ultrafiltration with Amicon Ultra-0.5 mL centrifugal filter units. The later method was used to enhance the recovery of SMX.NO-modified MPO.

For confirmation of the haptenation before protein purification using reversed phase chromatography, an aliquot of the modified protein was run on a SDS-PAGE gel as described above, Coomassie Blue staining performed, and the band was cut out and digested with trypsin. The peptides were extracted, and they were fractionated on the off-line reversed phase column prior to spotting on nitrocellulose and Western blotting to confirm drug-protein modification. In all, 30 fractions were collected between 11-40 min and Western blotting was performed using an anti-SMX antibody to identify fractions containing SMX.NO-modified MPO-derived peptides. In addition, adducts were purified and concentrated using the ultra-filtration technique.

After 1 hour of incubation, the mixture of MPO and SMX.NO was transferred to the Amicon Ultra-0.5 mL centrifugal filter unit and washed with either PBS or PBS containing an excess of GSH. In all, five washes (W1-W5) were carried out using either PBS or PBS containing GSH (1 mM). Washes were performed at 4°C and 14,000*g* for 30 min. After the fifth wash, a Bradford assay was used to quantify the amount of SMX.NO-modified MPO generated as previously described in section 2.3.7.

6.3.11 LC-MS/MS analysis of SMX.NO-modified MPO peptide/recombinant MPO protein

SMX.NO-modified MPO adducts and the synthetic peptide adducts generated as described above were analysed using LC-MS/MS. In each case, the degree of SMX.NO/MPO or SMX.NO/MPO peptide haptenation was determined. Details of the LC-MS/MS analysis are described in section 2.3.12.

6.3.12 LC-tandem mass spectrometric analysis of residual SMX.NO and its degradation products

6.3.12.1 Sample processing

To confirm the removal of residual SMX.NO from preparations of SMX.NO-modified MPO, filtrates of SMX.NO-modified MPO protein were analysed. Authentic standards of SMX, SMX.NHOH and SMX.NO were first analysed. SMX was dissolved in water-methanol (75:25, v/v; 500 μg/mL) and the resulting solution was diluted with methanol as required. SMX.NHOH dissolved in DMSO was diluted with methanol (1:25, v/v; 500 μg/mL). SMX.NO dissolved in DMSO (13.1 mg/mL) was diluted with ACN (LC-MS grade; 500 μg/mL), and the resulting solution was diluted serially with ACN for immediate analysis. Aliquots of these solutions (1.0 μL for chromatographic assessments; 10 μL for estimation of the sensitivity of SMX.NO detection), the MPO ultrafiltration washes (10 μL) and the supernatants of MPO solutions that had been deproteinized with ACN immediately before the analyses (10 μL) were injected onto the HPLC column without further treatment.

6.3.12.2 Analyses of ultrafiltration washes and deproteinized solutions

Aliquots of the standard solutions, ultrafiltration washes and deproteinized solutions were chromatographed at room temperature on an Agilent 5-μm

Zorbax Eclipse XDB-C8 column (150 mm × 4.6 mm; Agilent Technologies, Santa Clara, CA, USA) by gradient elution with acetonitrile in 0.05% formic acid. The column was protected with an Agilent Zorbax C18 Reliance cartridge guard-column.

Eluent was delivered by a PerkinElmer series 200 HPLC system (pump and autosampler; PerkinElmer, Norwalk, CT, USA). The eluent flow rate was 1.0 mL/min. The column was connected to the Turbo V electrospray source of an API 4000 Qtrap hybrid quadrupole mass spectrometer (AB Sciex, Warrington, UK) via a Valco flow-splitting T-piece. The split flow of eluate was approx. 150 µL/min. Synthetic compounds and experimental analytes were eluted with the following gradient of acetonitrile (LC-MS grade; Fisher Scientific) in 0.05% (v/v) formic acid: 15%→60% over 20 min, 60% for 1.0 min, 60%→15% over 0.1 min, 15% for 4.9 min.

Table 6.1.Typical retention times (R_t) of the analytes

Analyte	R_t (min)
SMX.NHOH	8
SMX	9
SMX.NO	15
Azoxy SMX	18

The operating parameters of the mass spectrometer for full scanning operation: source temperature, 450°C; ionspray (electrospray capillary) voltage, 4,500 V; scanning, *m/z* 100-1,000 in 5 s (other relevant parameters, as for MRM

operation). MRM operation: source temperature, 450°C; ionspray (electrospray capillary) voltage, 4,500 V; desolvation potential (DP), 100 V; entrance potential (EP), 10 V; CAD gas setting, 5; collision energy (CE), 41 eV; collision exit potential, 3 V; channel dwell time, 150 ms; curtain gas setting, 15; spray gas (Gas-1) setting, 50; heater gas (Gas-2) setting, 50. The instrument was set up in positive-ion mode. MRM transitions were derived from individual LC-MS analyses of standard compounds, and were not optimized. The GSH-containing washes and the PBS washes analysed separately were sampled in reverse order, i.e. starting with W5, to avoid/minimize sample contamination by 'carry over'.

6.3.13 T-cell proliferation assay

Thymidine proliferation assay was used to determine the immunogenicity of SMX.NO-modified MPO/MPO derived peptides. SMX.NO-specific T-cell clones (5 × 10^4 cells, 50 µL) generated from SMX-hypersensitive patients (chapter 3) were co-cultured with irradiated autologous EBV-transformed B-cells (1 × 10^4 cells) in the presence of either unmodified or SMX.NO-modified MPO (170 µg/ml, 100 µL). SMX.NO (50 µM, 100µL) was used as positive control. The proliferation assay was set up in duplicate in a 96-well U-bottom plate. Plates were incubated for 48 h, [^{3}H]-thymidine (0.5 µCi) added for the final 16 h of incubation and T-cell proliferation evaluated using a scintillation counter. T-cell proliferation was calculated as the mean of duplicate wells. A parallel proliferation assay was conducted to determine the minimum stimulatory concentration of SMX.NO (0.5 – 100 µM). Furthermore, T-cell responses from the filtrate after PBS washes (W1-W5) were measured.

6.3.14 Cation exchange chromatography

SMX.NO-modified synthetic MPO peptides were subjected to ion exchange chromatography using a Polysulfoethyl A strong cation-exchange column (200 × 4.6 mm, 5 μm, 300 Å; Poly LC, Columbia, MD). The samples were diluted to 4 mL in 10 mM KH_2PO_4/25% acetonitrile (solvent A), pH 3, prior to loading onto the column. Peptides were eluted using a gradient from 0-15% solvent B (1 M KCl in 10 mM KH_2PO_4/25% acetonitrile, pH 3) in 45 min and 15-50% solvent B in 15 min at a flow rate of 1 mL/min, and 2 mL fractions were collected. The fractions were evaporated to dryness in a SpeedVac, and they were desalted using a macroporous C_{18} High-Recovery reversed phase column (4.6 × 50 mm, Agilent Technologies, Santa Clara, CA, USA) installed on a Vision workstation (AB Sciex) before being dried once more. The peptides were resuspended in 0.1% formic acid (10 μL) just prior to LC-MS/MS analysis.

6.4 Results

Initial experiments involved the characterization of adducts generated from synthetic MPO peptides and SMX.NO followed by the determination of the immunogenicity of SMX.NO-modified peptides. In subsequent experiments, recombinant MPO was modified with SMX.NO with the aim of increasing the recovery of the SMX.NO-modified hapten carrier.

6.4.1 Evidence of SMX.NO/MPO peptide and SMX.NO/MPO protein adduction

SMX.NO modification of either MPO-derived peptides or recombinant full-length MPO was determined using both Western blotting and LC-MS/MS analysis.

6.4.1.1 Western blot analysis of SMX.NO/MPO haptenation

The limit of MPO detection using Western blotting was determined to be 0.1 mg/ml (figure 6.3A). MPO/SMX.NO haptenation was weakly detected at 1:1 molar ratio. A higher degree of protein modification was observed with increased molar ratios (1:5 and 1:10), as illustrated in figure 6.3 B and C. Furthermore, binding of SMX to MPO was dependent on the concentration of SMX, and hydrogen peroxide was critical for the conversion of SMX to SMX.NO (figure 6.3D). Finally, the formation of SMX.NO/protein adduct was time-dependent, with optimum MPO haptenation occurring at between 30-60 min (figure 6.3E). A time point of 1 hour was used for subsequent MPO haptenation.

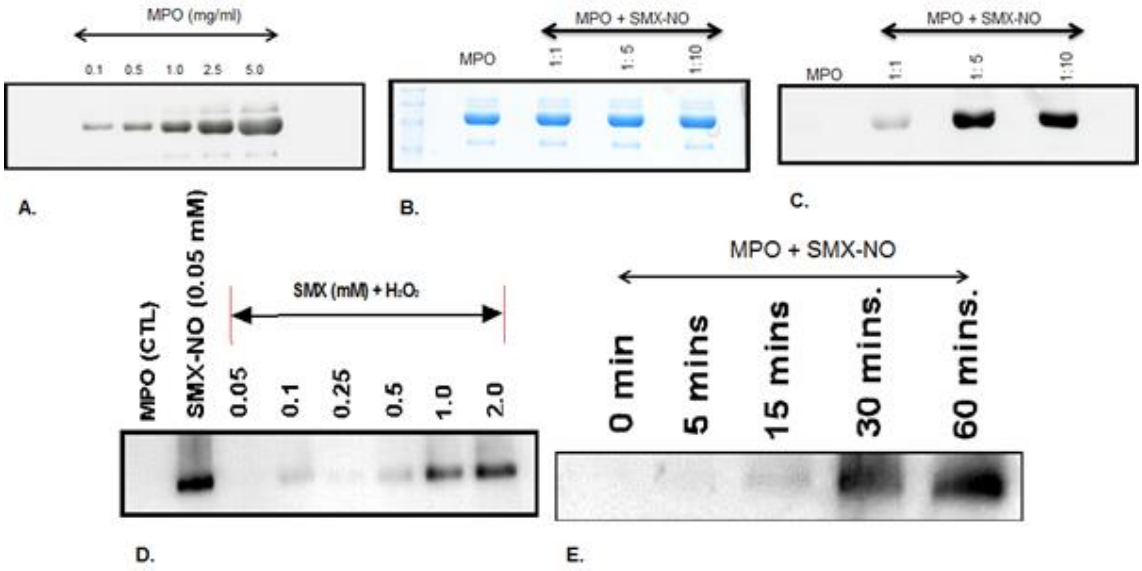


Figure 6.3 Western blot analyses of SMX.NO/MPO adduct. **(A)** Limit of detection for recombinant MPO protein. Dilutions of MPO (0.1-5.0 mg/ml) were processed, and separated on a 12% SDS-PAGE gel before Western blotting for MPO using an anti-MPO antibody. **(B)** Coomassie Blue staining of MPO used as loading control for Western blotting of SMX.NO/MPO adducts. **(C)** MPO was incubated with SMX.NO (1:1, 1:5 and 1:10 molar ratio) for 1 hour at 37°C. Samples were then processed, and separated using a 12% SDS-PAGE gel before Western blotting with an anti-SMX rabbit antibody. **(D)** MPO (1.5 mg/ml) was incubated with or without SMX.NO (50 µM) or with various concentrations of SMX (0.05-2 mM) in the presence of hydrogen peroxide (10 µM) for 1 hour at 37°C. Samples were then processed, and separated using a 12% SDS-PAGE gel before Western blotting with an anti-SMX rabbit antibody. **(E)** MPO (1.5 mg/ml) was incubated with SMX.NO (50 µM) in a 1:5 molar ratio for 0-60 min. Samples were processed, and separated on a 12% SDS-PAGE gel before Western blotting with an anti-SMX rabbit antibody

6.4.1.2 LC-MS/MS analysis of SMX.NO-modified MPO-derived peptide

The characteristics of the MPO-derived peptides synthesised by Invitrogen are given in table 6.2 below. MPO2 and MPO3 showed 80.0% and 96.3% homology respectively when compared with MPO1. SMX.NO haptenation was observed in various degrees on MPO1 and MPO3 but not MPO2 (no cysteine residue).

Table 6.2-Chemical properties of MPO-derived peptides: red letters in the peptide sequences indicate alanine substitutions in the synthesis of MPO2 and MPO3.

MPO derived Peptide	Peptide Sequence	Theoretical molecular weight	Observed molecular weight	Physical appearance	Percentage purity
MPO 1	ADCIPFFRSCPACPG	1583.86	1583.00	White to off white powder	97.38%
MPO 2	ADAIPFFRSAPAAPG	1487.69	1487.81	White to off white powder	96.22%
MPO 3	ADAIPFFRSCPAAPG	1519.75	1519.80	White to off white powder	95.30%

LC-MS/MS analyses of off-line reversed phase HPLC fractions collected at 34, 35 and 36 min are illustrated in figure 6.4. The LC-MS/MS characterization of SMX.NO-modified MPO1 indicates the presence of the N-hydroxysulphinamide residue on Cys309 and the formation of an inter-chain disulphide bond (figure 6.5). The panels on the left contain extracted ion chromatograms (XIC) that revealed the formation of both intra- and inter-peptide disulphide bonds.

Native MPO, which is a tetramer (see figure 6.2), has both types of disulphide bridge: each half molecule has one inter-chain bridge and six intra-chain bridges (Zeng and Fenna 1992). It is very difficult to determine from the MS/MS spectra the exact position of the disulphides, but the presence of at least three separate chromatographic peaks suggested that multiple disulphide bonds had formed. Similarly, a peptide modified with the N-hydroxysulphinamide residue (283 amu) plus an intra-peptide disulphide was detected (middle panels), but the presence of multiple chromatographic peaks suggested the positions of these modifications varied. The panels on the right are zoom-in presentations of the

middle panels to show the multiple chromatographic peaks. Subsequent attempts to interpret the signals are shown in figure 6.7.

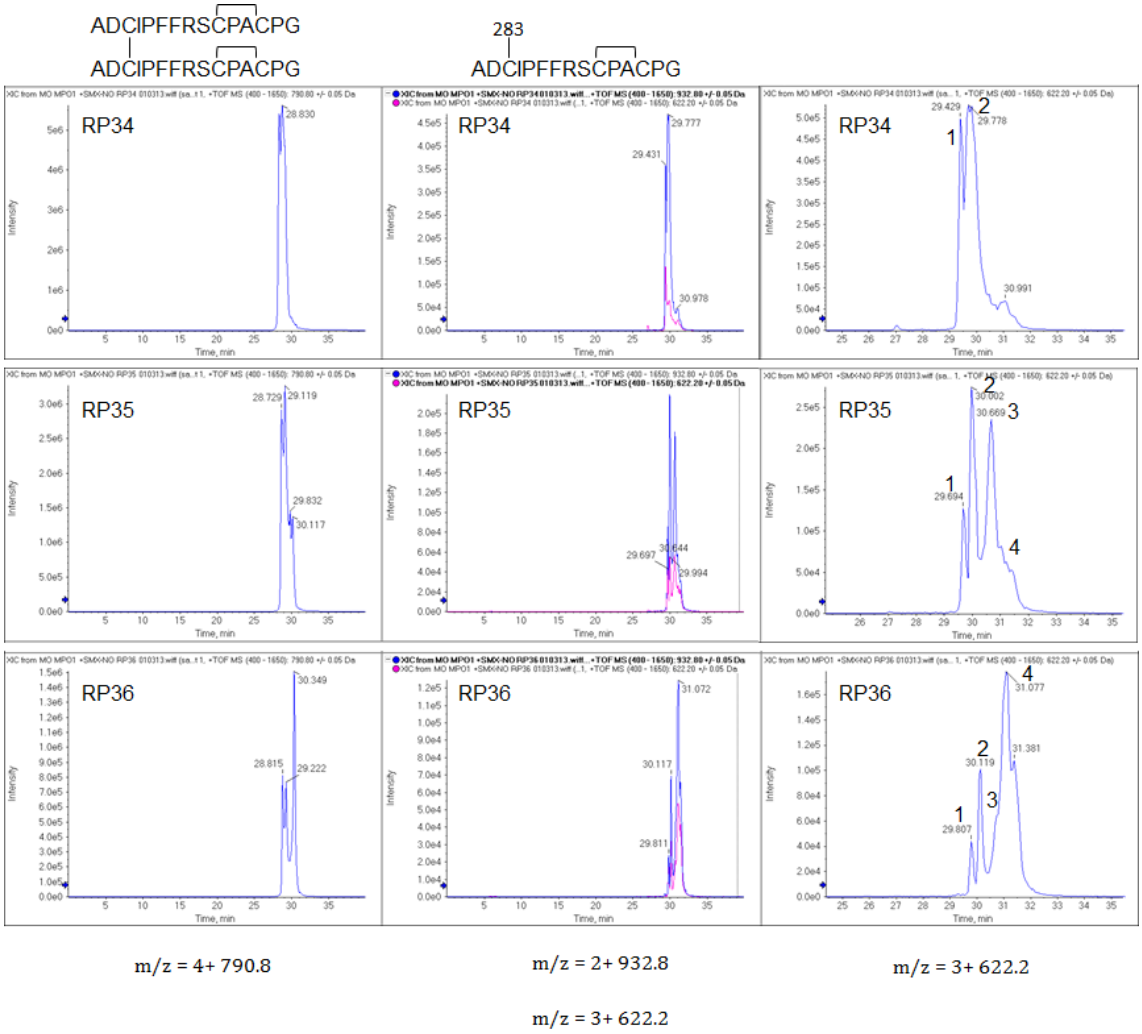


Figure 6.4 Cation exchange traces. Off-line reversed phase purification of SMX.NO-modified MPO1 peptide. Samples eluting at 34, 35 and 36 min were collected for analysis.

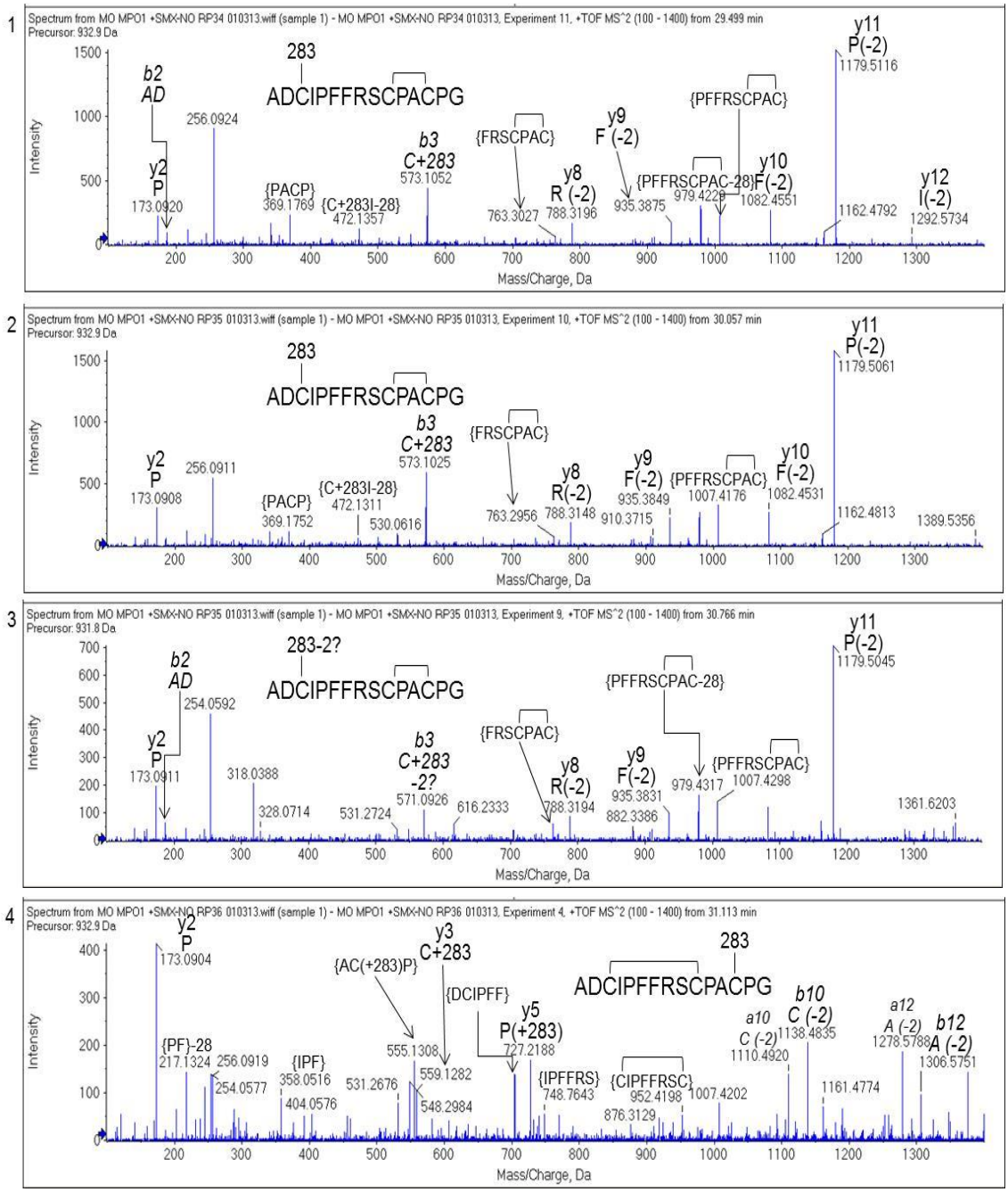


Figure 6.5 Mass spectrometric characterization of SMX.NO-modified MPO1 peptide. Spectrum 1 and Spectrum 2 appear to be the same peptide, most likely rotational isomers. Spectrum 3 revealed a loss of 2 amu from the drug adducts (internal disulphide bridge between two cysteine residues resulted in loss of 2×hydrogen atoms). Spectra 1-4 represent reversed phase fractions collected at 34, 35 and 36 min.

Because of the complexity of the analysis due to disulphide bond formation in peptide MPO1, an additional peptide was synthesised in which two of the cysteine residues (Cys309, Cys319) had been substituted with alanine residues (MPO3). The analysis of SMX.NO/MPO3 haptenation is illustrated in figure 6.6 and figure 6.7. MPO3 was modified with nitroso-SMX, fractionated by off-line RP-HPLC and the fractions were assessed by MALDI-MS. This assessment identified peptide fractions which were taken forward for full LC-MS/MS analysis. Reversed phase fractions were collected at 27, 28, 29 and 30 min.

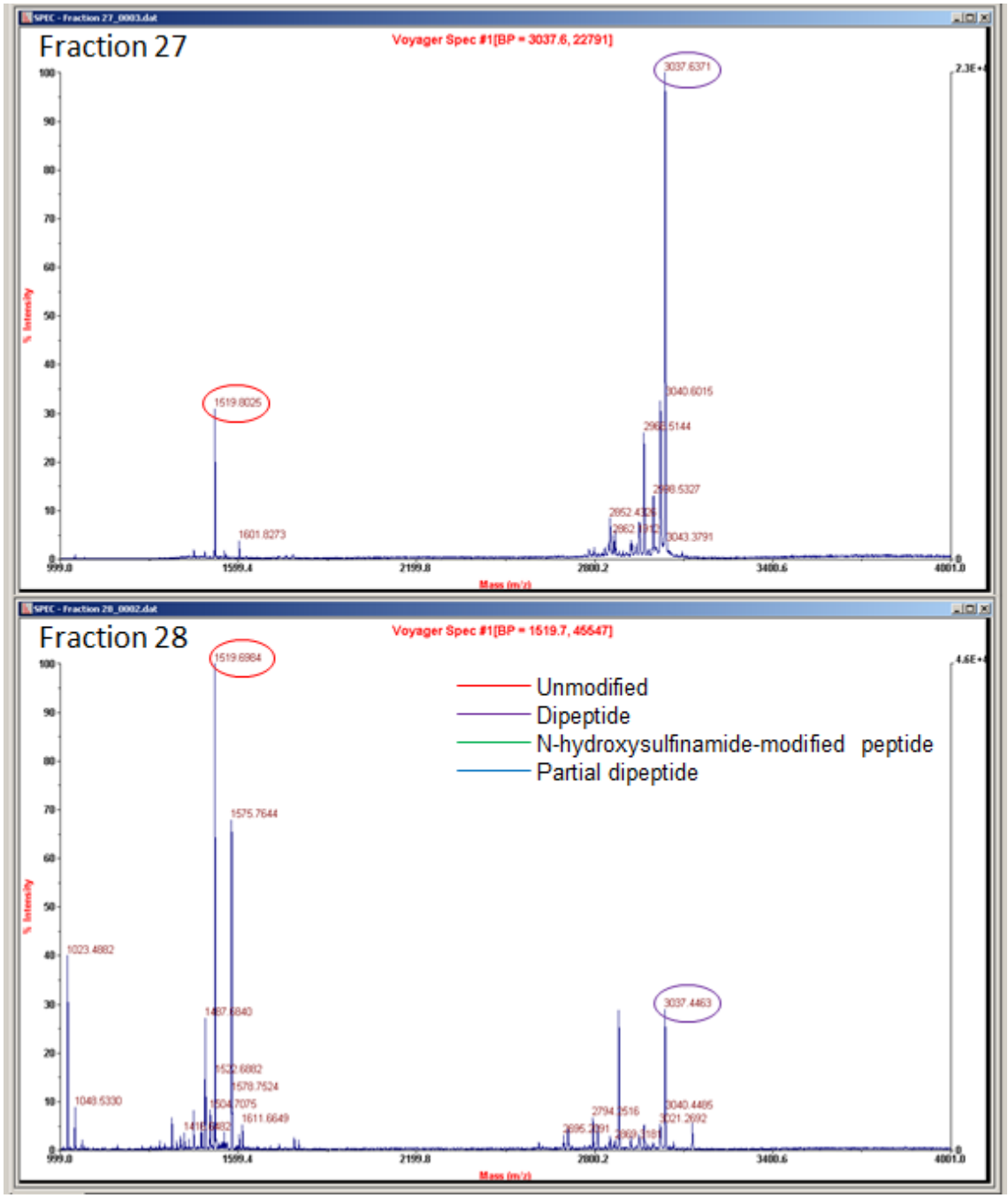


Figure 6.6 MALDI-MS analysis of SMX.NO-modified MPO3 fractionated by off-line RP-HPLC. The fractions were collected after 27 min and 28 min (upper and lower panels respectively). Mass of unmodified peptide = 1519.80, mass of dipeptide = 3037.66.

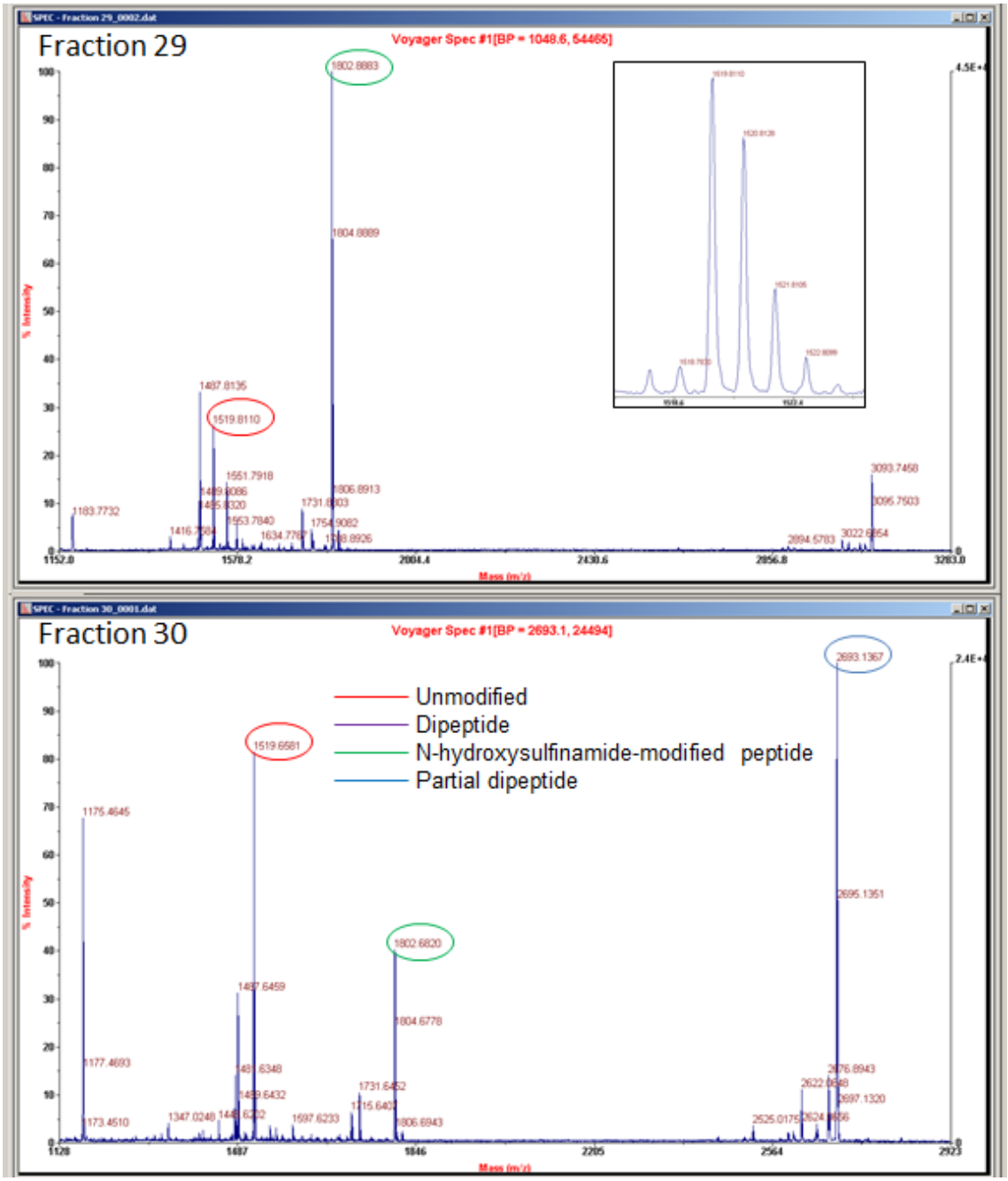
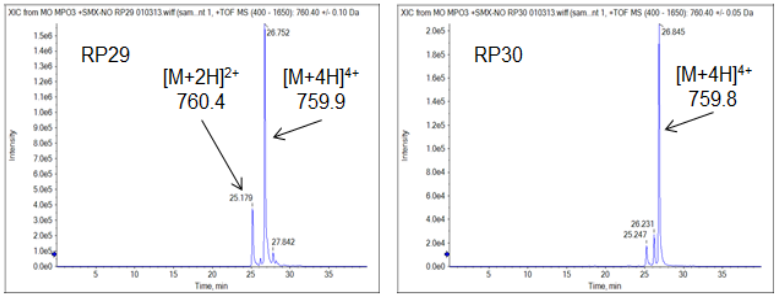
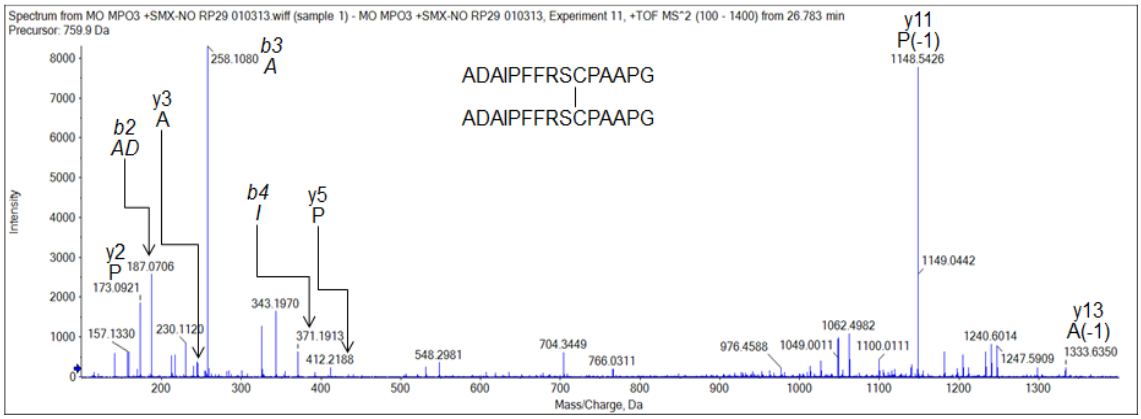


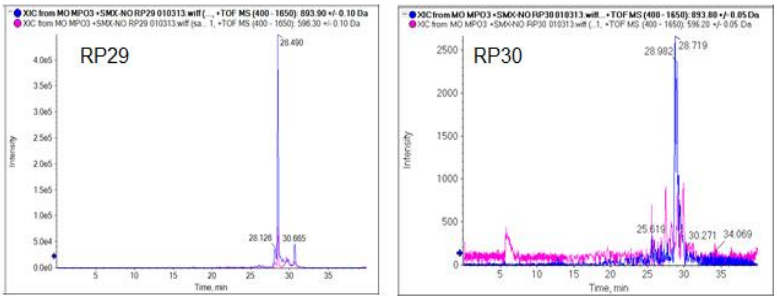
Figure 6.7 MALDI-MS analysis of SMX.NO-modified MPO3 fractionated by off-line RP-HPLC. The fractions were collected after 29 min and 30 min (upper and lower panels respectively). Calibrated masses were determined as follows: unmodified peptide = 1519, N-hydroxysulphinamide-modified peptide = 1802.88, partial dipeptide = 2696.16.

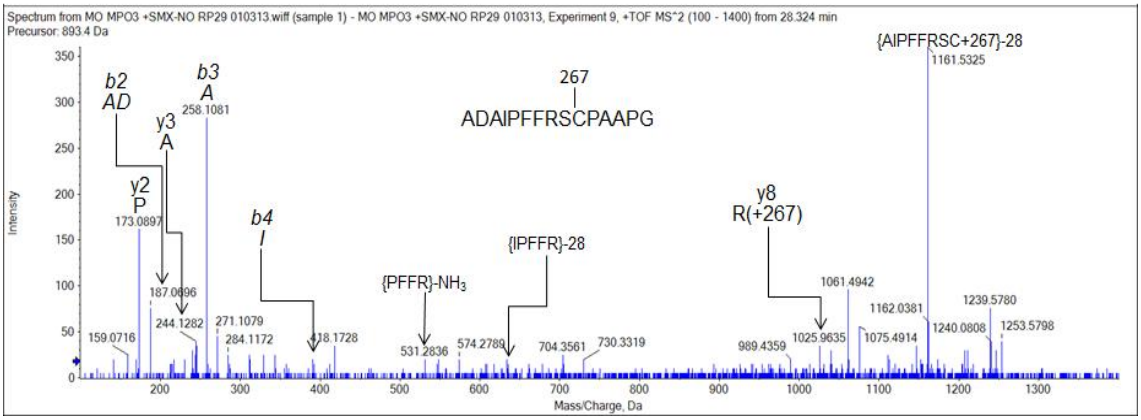
A.


RP29
[M+2H]2+
760.4
[M+4H]4+
759.9
RP30
[M+4H]4+
759.8


Spectrum from MO MPO3 +SMX-NO RP29 010313.wiff (sample 1) - MO MPO3 +SMX-NO RP29 010313, Experiment 11, +TOF MS^2 (100 - 1400) from 26.783 min
Precursor: 759.9 Da
ADAIPFFRSCPAAPG
ADAIPFFRSCPAAPG
b3 A
y11 P(-1)
1148.5426
b2 AD
y3 A
b4 I
y5 P
y2 P
187.0706
173.0921
343.1970
371.1913
412.2188
1149.0442
y13 A(-1)
1333.6350
Intensity
Mass/Charge, Da

B.


RP29
RP30


Spectrum from MO MPO3 +SMX-NO RP29 010313.wiff (sample 1) - MO MPO3 +SMX-NO RP29 010313, Experiment 9, +TOF MS^2 (100 - 1400) from 28.324 min
Precursor: 893.4 Da
ADAIPFFRSCPAAPG
267
{AIPFFRSC+267}-28
1161.5325
b2 AD
b3 A
258.1081
y3 A
y2 P
173.0897
187.0696
b4 I
{IPFFR}-28
{PFFR}-NH3
y8 R(+267)
1061.4942
1162.0381
1239.5780
1240.0808
1253.5798
1025.9635
1075.4914
989.4359
159.0716
244.1282
271.1079
284.1172
418.1728
531.2836
574.2789
704.3561
730.3319
Intensity
Mass/Charge, Da

C.

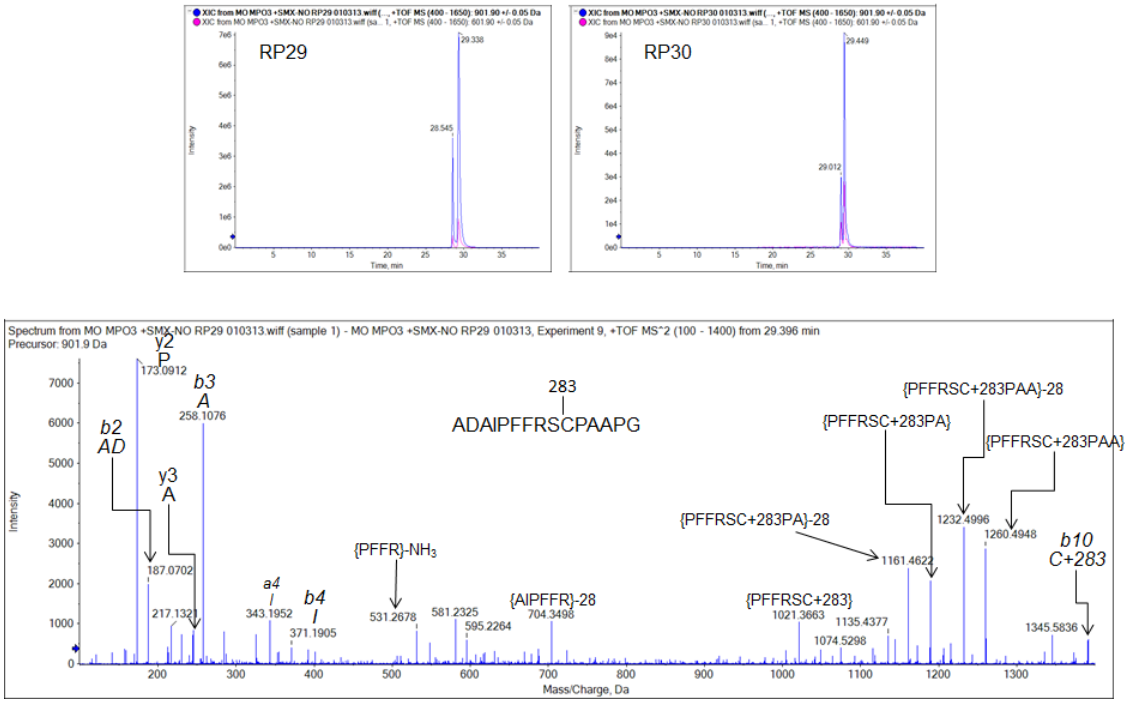


Figure 6.8 Representative MS^3 spectra and mass chromatograms of characteristic fragment ions from SMX.NO-modified MPO3 peptides following reversed phase HPLC for samples collected after 29 min and 30 min. **(A)** Characteristic internal fragment ions consistent with formation of a disulphide **(B)** Weak characteristic internal fragment ions suggestive of sulphinamide adduct (267 amu residue) formation. **(C)** Convincing internal fragment ion to confirm the presence of N-hydroxysulphinamide adduct (m/z = 2+ 901.8, 3+ 601.6)

Spectra of SMX.NO-modified MPO3 showed the formation of a dipeptide which complicated the analysis of fragment ions (figure 6.8A). Although the spectrum was weak, there was evidence of a sulphinamide adduct (figure 6.8B) and for an N-hydroxysulphinamide adduct (figures 6.7 and 6.8C). No peptide with the N-hydroxysulphonamide adduct was detected.

Since off-line RP-HPLC failed to separate modified from unmodified peptide, a different off-line fractionation was tried, namely cation exchange chromatography (CEX). Again, cation exchange chromatography failed to separate modified from unmodified MPO3, but at least the dipeptide eluted

much later in the chromatographic run (figure 6.9). Cation exchange chromatography also revealed the presence of many variants of the MPO3 peptide formed by amino acid deletions, additions, substitutions and chemical modification during synthesis, further complicating the interpretation of spectra generated (figure 6.10).

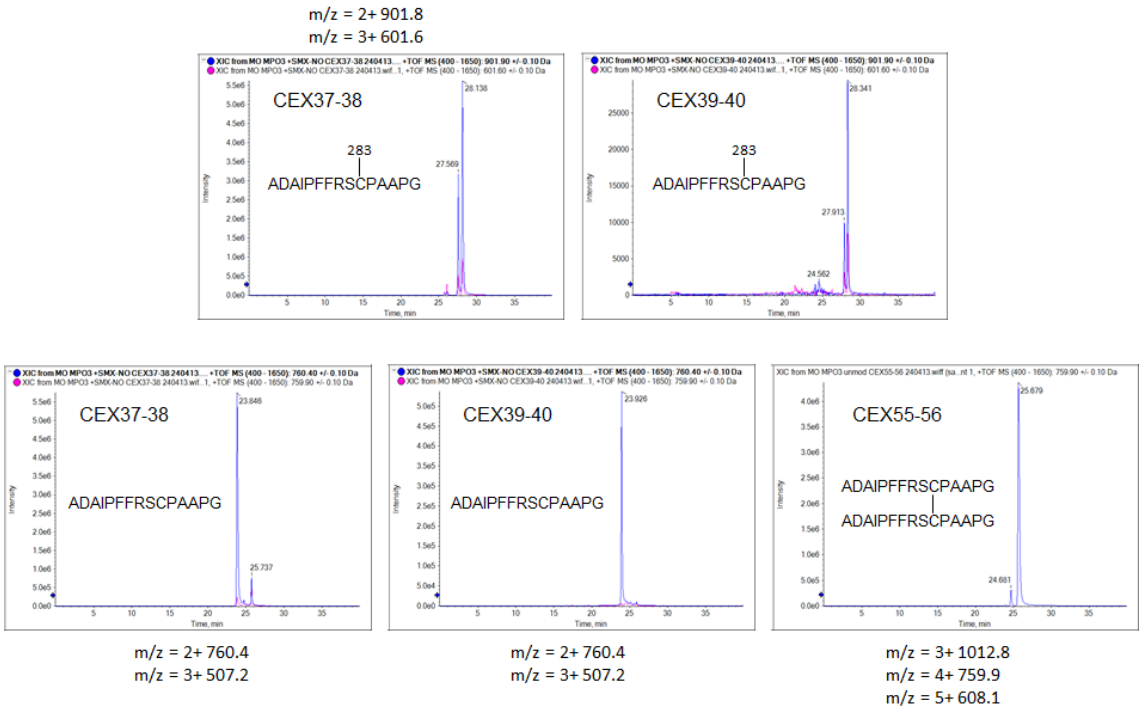


Figure 6.9 Cation exchange chromatography aimed to purify MPO3/SMX.NO adducts for fractions collected between 37-38, 39-40 and 55-56 min. The 283-amu residue is an N-hydroxysulphinamide adduct.

Figure 6.10 Variations of MPO3 peptide formed by amino acid deletions, additions, substitutions and chemical modification during synthesis.

The concentration of the semi-purified modified peptide was estimated using the unmodified peptide to generate a standard curve. A dilution series of the MPO3 peptide was analysed by LC-MRM-MS in a QTRAP 5500. Briefly, the peptide was diluted in HSA in order to prevent loss through adhesion to plastic vials and tubes. Aliquots of 1 µL of the diluted peptide were delivered into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (AB Sciex) by automated in-line liquid chromatography (U3000 HPLC System, 5 mm C18 nano-precolumn and 75 µm ×15 cm C_{18} PepMap column [Dionex, California, USA]) via a 10 µm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 2% ACN/0.1% FA (v/v) to 50% ACN/0.1% FA (v/v) in 60 min was applied at a flow rate of 300 nL/min. The ionspray potential was set to 2,200-3,500 V, the nebuliser gas to 19 and the interface heater to 150^{o}C.

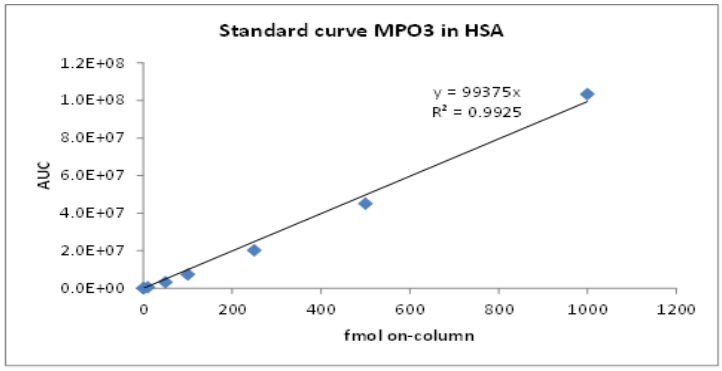
MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity, they were optimised for collision energy and collision cell exit potential, and dwell time was 50 ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified

peptides, with Q1 set to unit resolution, dynamic fill selected and dynamic exclusion for 20 s. The MRM transitions were designed using the *m/z* values of the unmodified and modified MPO3 peptide in combination with the dominant fragment ion masses observed for each (see table 6.3).

Table 6.3- MRM transition values for the calibration of MPO3 adduct.

Q1	Q3	Dwell time	ID	CE
760.4	977.5	50	ADAIPFFRSCPAAPG 2+ a9	30
760.4	531.3	50	ADAIPFFRSCPAAPG 2+ (PFFR-NH3)	50
760.4	258.1	50	ADAIPFFRSCPAAPG 2+ b3	40
760.4	173.2	50	ADAIPFFRSCPAAPG 2+ y2	30
901.9	704.4	50	ADAIPFFRSCPAAPG +SMX 2+ (AIPFFR-28)	30
901.9	531.3	50	ADAIPFFRSCPAAPG +SMX 2+ (PFFR-NH3)	50
901.9	258.1	50	ADAIPFFRSCPAAPG +SMX 2+ b3	40
901.9	173.2	50	ADAIPFFRSCPAAPG +SMX 2+ y2	30

A standard curve of ion count versus femtomoles of peptide loaded on the column was generated (figure 6.11), and the concentration of the modified peptide was determined by adjusting the MRM transitions to account for the presence of adduct with the mass of 283 amu (N-hydroxysulphinamide). Only 1.912 µg of SMX.NO-modified MPO3 was recovered and utilised in biological assay.



Mod sample	AUC	fmol/µl at 1:1000	fmol/µl neat	µmol/ml	mg/ml
Unmod MPO3	8.063E+05	8.114	8113.711	0.008	0.012
Mod MPO3	1.279E+06	12.870	12870.440	0.013	0.020

Total peptide in 60µl = 1.912µg

Figure 6.11 Quantification of unmodified and SMX.NO-modified MPO3 peptide using calibration curve of MPO in a tryptic digest of HSA.

Although SMX.NO-modified MPO3 failed to stimulate T-cell proliferation (figure 6.12), it was not clear at this point if the non-responsiveness of SMX.NO-specific cells was a direct result of the low concentration of drug-modified MPO3 adduct incubated in the proliferation assay or a lack of recognition of the antigenic epitope by the drug-specific TCCs. A number of studies have used peptide concentrations between 50-100 µg/mL. Furthermore, experiments where MPO3 (2 mg/ml) was incubated with SMX.NO (5-50 µM) for 1 hour followed by addition of excess of GSH (1 mM) for 30 min to reduce free SMX.NO failed to activate drug-specific TCCs (figure 6.12). Hence, the decision was made to modify the full-length recombinant MPO.

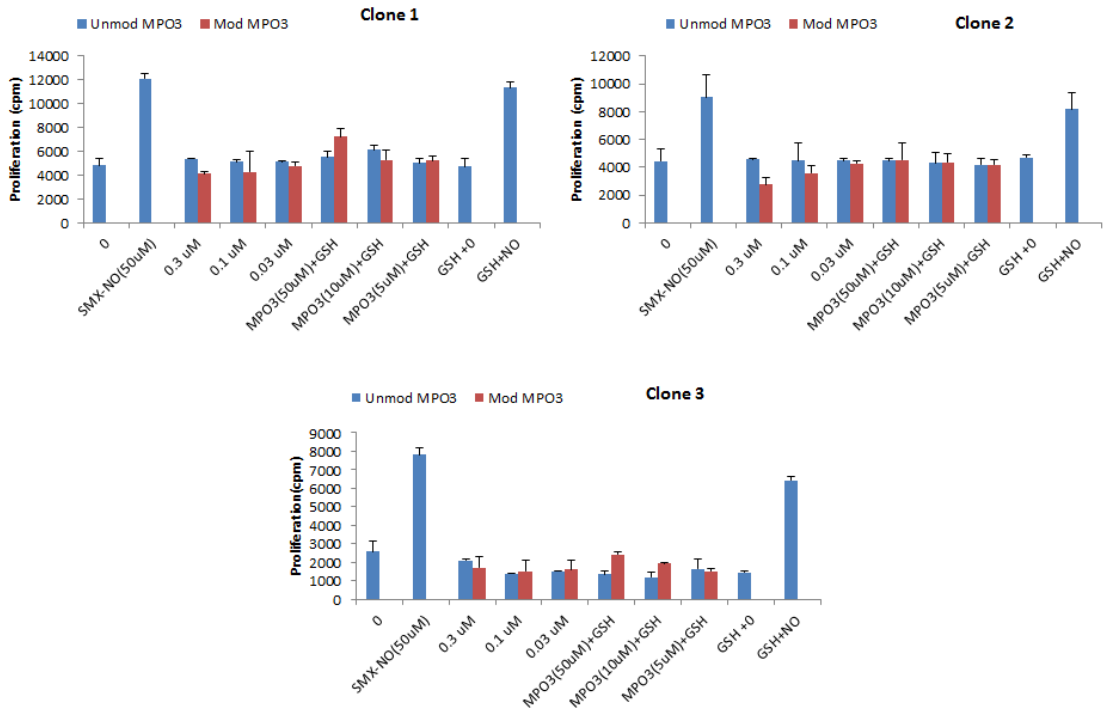


Figure 6.12 T-cell proliferative responses to SMX.NO-modified MPO3 peptide. SMX.NO-specific T-cell clones (5×10^4 cells, 50 µL) generated from an SMX hypersensitive patient were co-cultured with autologous APCs (1×10^4 cells) with either unmodified or SMX.NO-modified MPO3 peptide (0.03-0.3 µM) in a 96-well U-bottom plate. In other experiments, MPO3 (5-50 µM) was incubated with SMX.NO (50 µM) for 1 hour. After 1 hour, GSH (1 mM) was used to neutralize free SMX.NO. SMX.NO (50 µM) and culture medium were used as positive and negative controls, respectively. The plate was incubated at 37°C, under an atmosphere of 95%O_2/5% CO_2 for 48 h. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 h of incubation and T-cell proliferation evaluated using scintillation counter.

6.4.1.3 LC-MS/MS analysis of SMX.NO-modified MPO

Subsequent experiments involved the attempted LC-MS/MS analysis of SMX.NO/MPO haptenation. The recombinant MPO protein that had been incubated with SMX.NO was analysed on a 12% SDS-PAGE gel to eliminate any free SMX.NO from the analysis of drug-protein adducts prior to tryptic digestion. MPO was incubated with SMX.NO for 1 hour at 1:10 molar ratio followed by gel electrophoresis, Coomassie Blue staining and in-gel tryptic digestion. LC-MS/MS analysis achieved 81% sequence coverage but no modification of MPO by SMX.NO was detected. Unsuccessful MS/MS searches for SMX.NO-modified MPO

included sulphinamide, *N*-hydroxysulphinamide, and *N*-hydroxysulphonamide adducts. Conversely, Western blot analyses of the SMX.NO/MPO adduct suggested MPO modification as illustrated in figure 6.13.

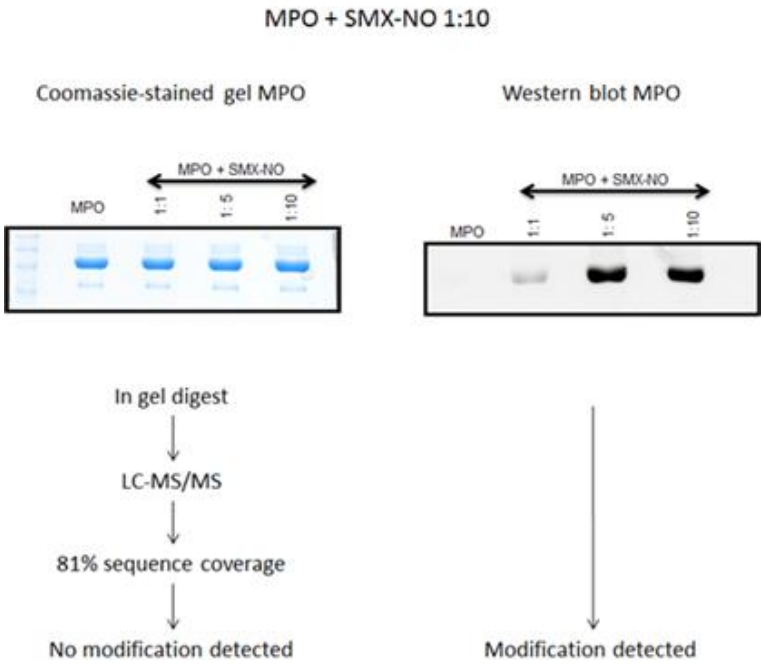


Figure 6.13 Schematic of steps and outcomes of SMX.NO/MPO adduct detection using LC-MS/MS and Western blotting techniques.

Since no SMX.NO-modified peptides were detected when the entire protein digest was analysed by LC-MS, the mixture of peptides was subjected to off-line reversed phase chromatography in order to enhance the sensitivity of MS detection and to simplify the data analysis (figure 6.14A). However, it still was not possible to identify modified peptides. Each fraction collected from the off-line reversed phase column was spotted onto a sheet of nitrocellulose and probed with anti-SMX antibody to determine which fraction(s) contained drug-modified peptides (figure 6.14B). The positive fractions were then exhaustively

analysed by LC-MS/MS and the data submitted to re-scripted protein analysis software (ProteinPilot 4, AB Sciex) in order to detect modification.

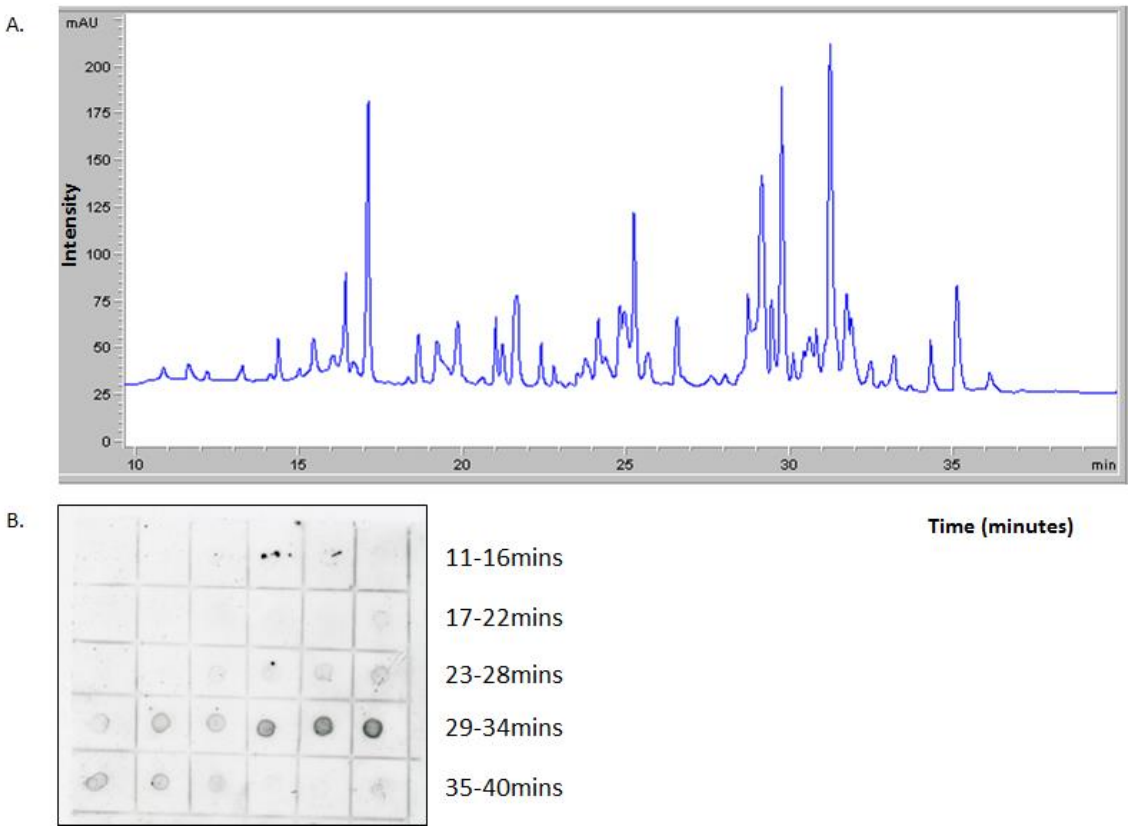


Figure 6.14 (A) Off-line reversed phase fractionation of SMX.NO-modified MPO-derived tryptic peptides. Fractions corresponding to prominent peaks detected at 280 nm were collected and analysed using LC-MS/MS. **(B)** Fractions were collected between 11-40 min, spotted on nitrocellulose membrane, followed by Western blot analysis for SMX.NO-modified MPO adducts.

Two SMX.NO-modified tryptic peptides were detected, but neither contained the Cys316 modification observed in the MPO3/SMX.NO adduct. N-hydroxysulphinamide adducts (with modifications on Cys309 and Cys398) were observed (figure 6.15). Cys316 contained a sulphenic acid modification, which, it was hypothesized, would enhance SMX.NO haptenation (see figure 6.1). A number of reactive metabolites can generate protein-sulphenic acid intermediates resulting in both functional and toxic cellular outcomes

(Kettenhofen and Wood 2010). However, it is possible that the extensive glycosylation of Asn323 by N-acetyl-glucosamine sterically blocks access to Cys316 by nitroso-SMX, preventing modification (figure 6.16). The patterns of the modifications of MPO, MPO1 and MPO3 by SMX.NO are quite complicated as illustrated on table 6.4.

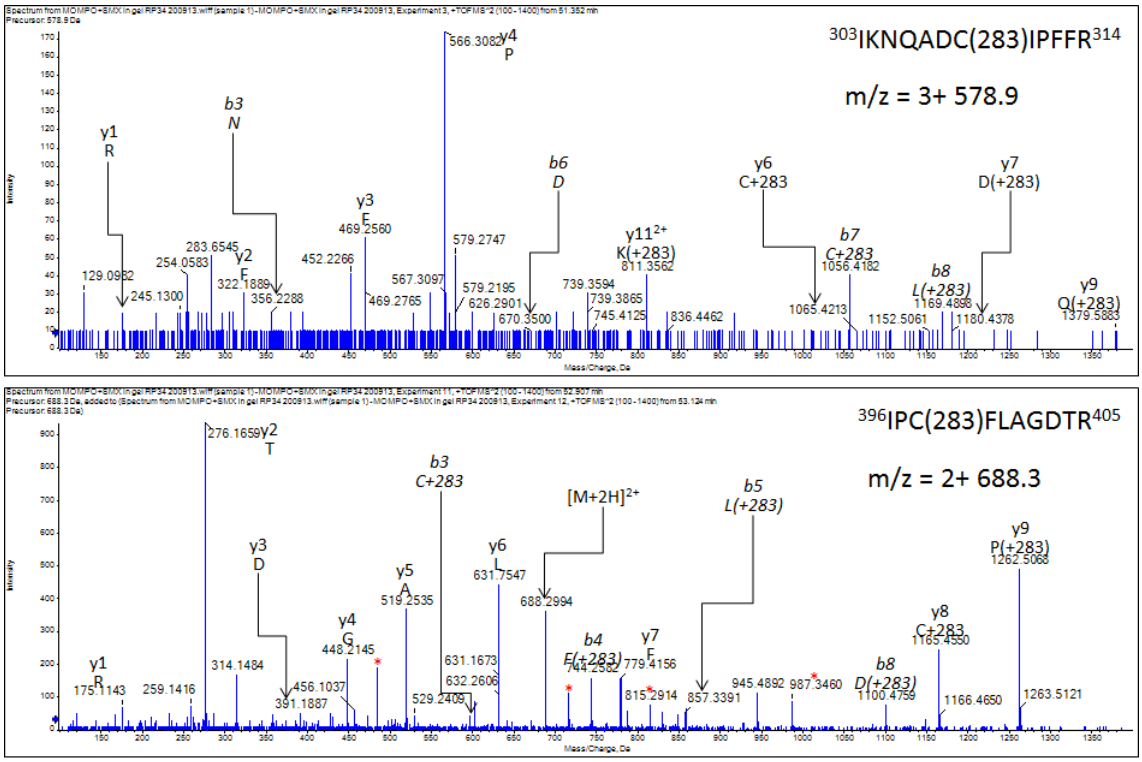


Figure 6.15 Representative MS^3 spectra of characteristic fragment ions from N-hydroxysulphinamide adduct. Two modifications by SMX.NO of MPO-derived tryptic peptides occurred on Cys309 and Cys398.

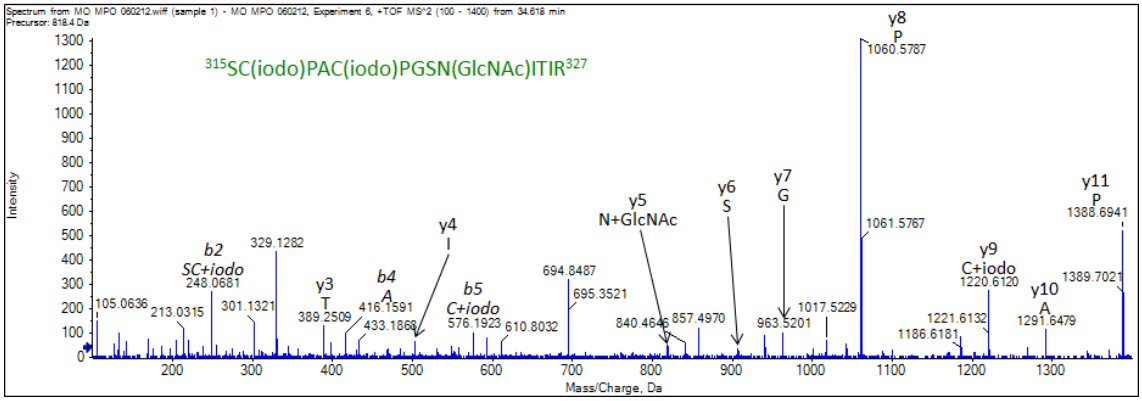


Figure 6.16 LC-MS/MS analysis of recombinant human myeloperoxidase with N-acetyl glucosamine residue on Asn323 (m/z = 2+ 818.4).

Table 6.4 Modification patterns of MPO and MPO peptides by SMX.NO.

MPO/MPO peptide	Cysteine residue (s) modified	Modification (s)
MPO1	309, 319	N-hydroxysulphinamide residue
MPO3	316	Sulphinamide, N-hydroxysulphinamide residue
Recombinant full length MPO	309, 398	N-hydroxysulphinamide residue

6.4.1.4 Purification of SMX.NO-modified MPO adducts using ultrafiltration

Amicon Ultra-0.5 mL centrifugal filter units were used to remove residual SMX.NO from preparations of modified MPO. Samples of protein on the column were washed 5 times (14,000 *g*, 4°C, 30 min) with either PBS or PBS containing excess GSH (1 mM). Excess GSH has been previously reported to reduce SMX.NO (Cribb et al. 1991).

LC-MS/MS analysis of all the washes and the supernatant obtained after the solution of the washed MPO was deproteinized were performed to exclude the presence of residual SMX.NO in the SMX.NO-modified MPO adduct. Standards of SMX, SMX.NHOH, SMX.NO, and the azoxy dimer that is invariably found in

synthetic preparations of SMX.NO, were resolved satisfactorily on a C8 column with a gradient of ACN in formic acid (0.05%, v/v) as in figure 6.17. Multiple reaction monitoring (MRM) transitions for the subsequent analyses were derived from individual LC-MS analyses. Table 6.5 records mass spectrometric parameters for the MRM detection of SMX and derivatives.

Table 6.5 -Mass spectrometric parameters for the multiple reaction monitoring (MRM) detection of SMX and derivatives.

Parameter	Set values of parameters			
	SMX	SMX.NOH	SMX.NO	Azoxy
MRM transition	*m/z* 254→156	*m/z* 270→99	*m/z* 268→238	*m/z* 519→268
Collision gas pressure setting	5	5	5	5
Collision energy (eV)	41	41	41	41
Desolvation potential (V)	100	100	100	100

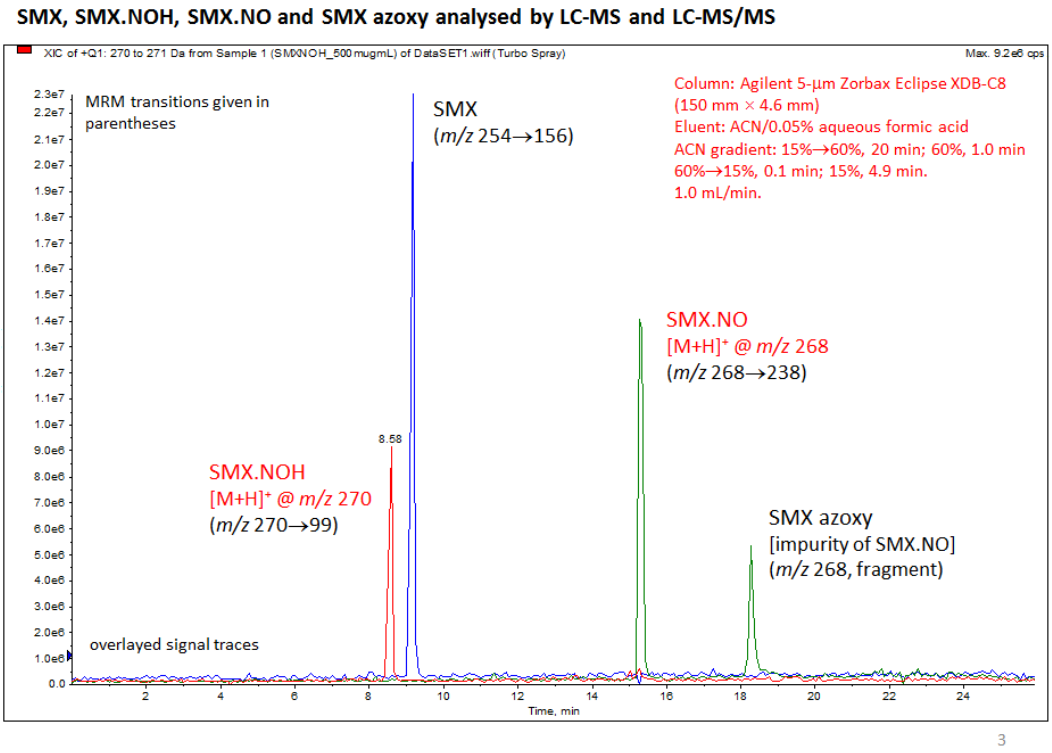
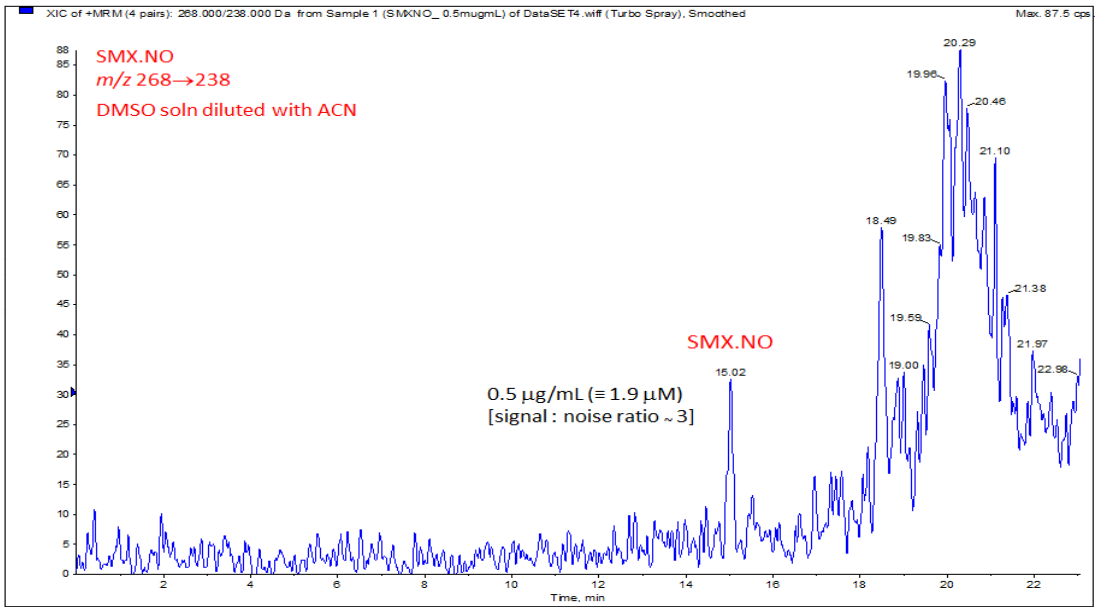


Figure 6.17 LC-MS and LC/MS/MS analysis of SMX.NHOH, SMX, SMX.NO and SMX azoxy standards illustrating their respective retention time following resolution using a C8 column with a gradient of ACN in formic acid (0.05%, v/v).

SMX.NO was dissolved in DMSO and serially diluted with ACN, and dilutions were analysed immediately by unoptimized MRM. The lower limit of SMX.NO detection was estimated to be 0.5 µg/ml (≡ 1.9 µM). SMX.NO was not detected at 0.25 µg/ml using this LC-MS/MS method (figure 6.18B). The ultrafiltration PBS washes (PB W1-W5) of MPO that had been incubated with SMX.NO were analysed by LC-MS/MS immediately after they were thawed from -20°C. W1 and W2 contained very high concentrations of SMX.NO (figure 6.19). Successive PBS washes contained lower concentrations of the nitroso (figure 6.20 and figure 6.21). W5 did not contain SMX.NO that could be identified with confidence; certainly the concentration was below 1.9 µM. SMX was detected in W1, W2 and W3. SMX.NHOH was only found in W2 and W3 washes. The azoxy was clearly

present in all of the washes up to and including W5. LC-MS/MS analysis of supernatant of the control MPO solution (protein not incubated with SMX.NO), consisting of PBS or PBS containing GSH, suggested no trace of SMX.NO (appendix ii). The control solutions of MPO were deproteinized with ACN (5 µl + 95 µl ACN). The supernatants were analyzed immediately. No signal corresponding to SMX.NO was detected in either supernatant. Furthermore, analyses of supernatants of solutions of SMX.NO-modified MPO adduct after either PBS or PBS+GSH washes (5^{th} wash) suggested no trace of SMX-NO (figure 6.23).

A. **LC-MS/MS analysis of SMX.NO: 0.5 µg/mL**



B. **LC-MS/MS analysis of SMX.NO: 0.25 µg/mL**

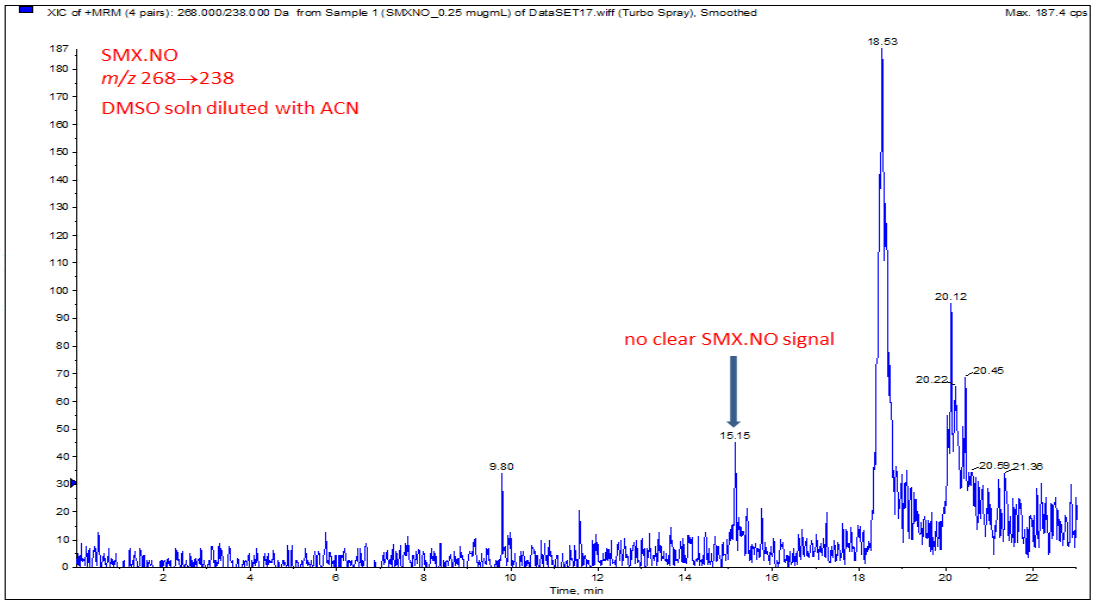


Figure 6.18 Limits of SMX.NO detection using LC-MS/MS. **(A)** Distinctive peak corresponding to SMX.NO was obtained at 0.5 µg/mL but not at 0.25 µg/mL **(B)**.

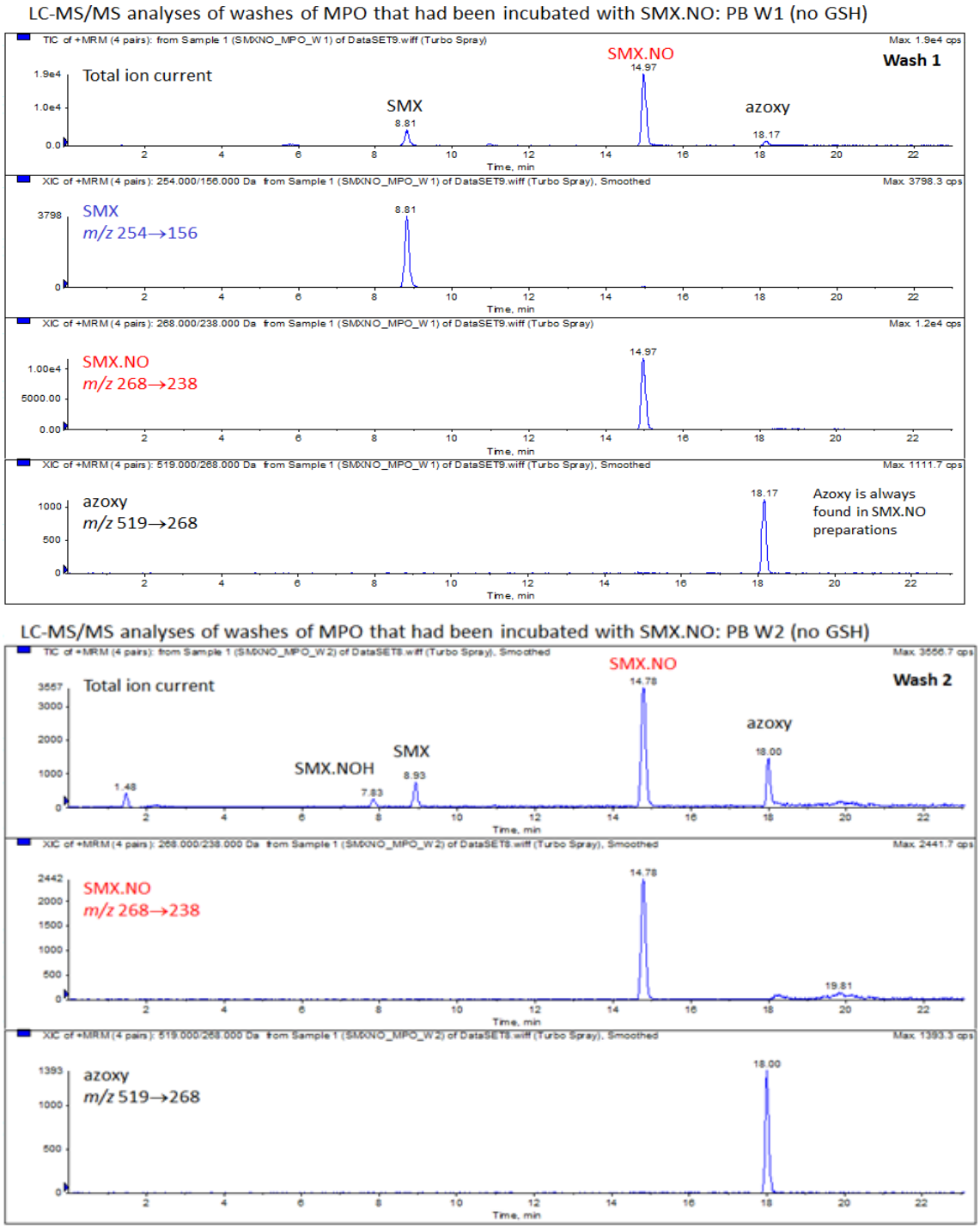


Figure 6.19 LC-MS/MS analysis of first and second washes (PB W1 and PB W2) of SMX.NO-modified MPO.

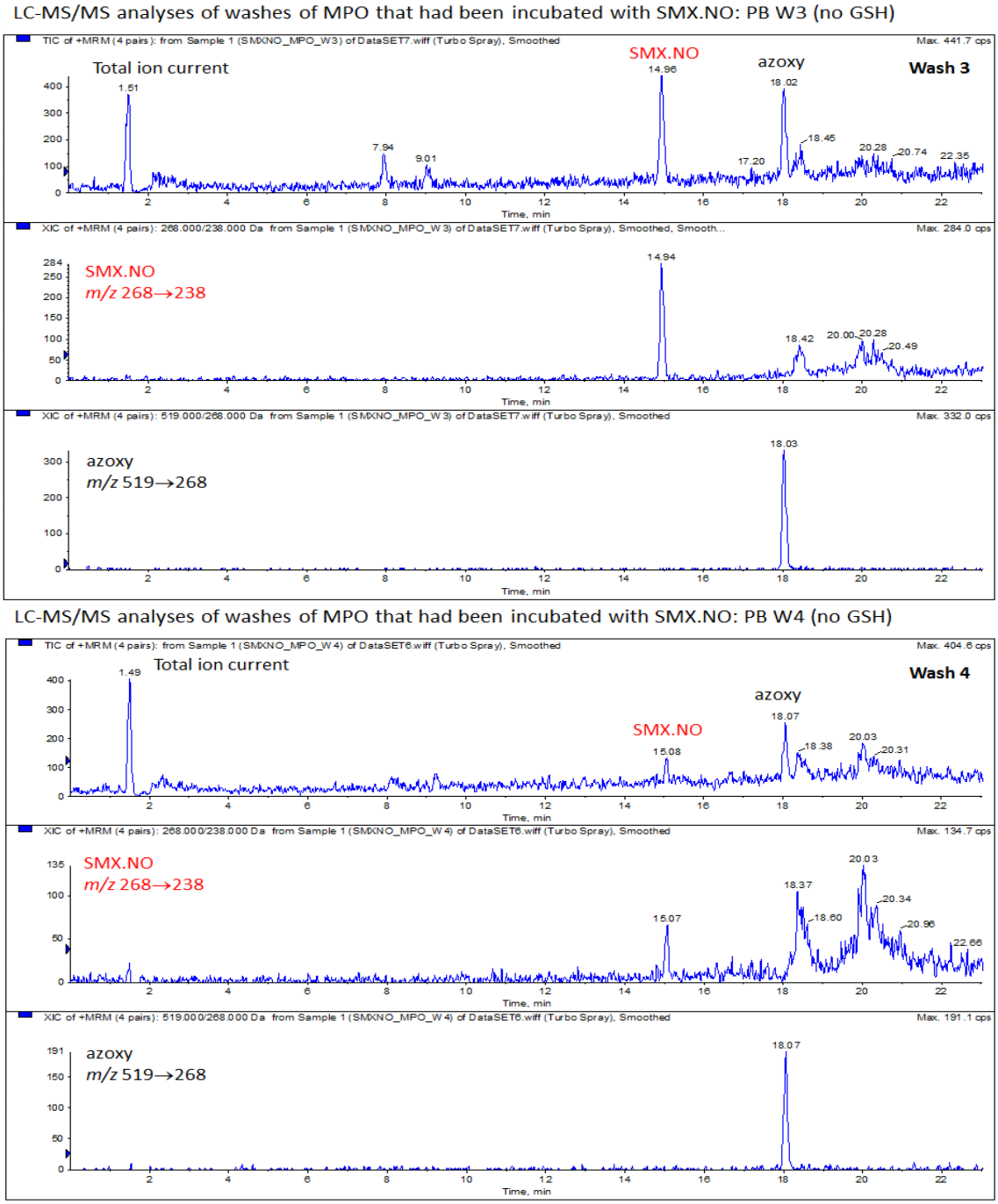


Figure 6.20 LC-MS/MS analysis of 3rd and 4th washes (PB W3 and PB W4) of SMX.NO-modified MPO.

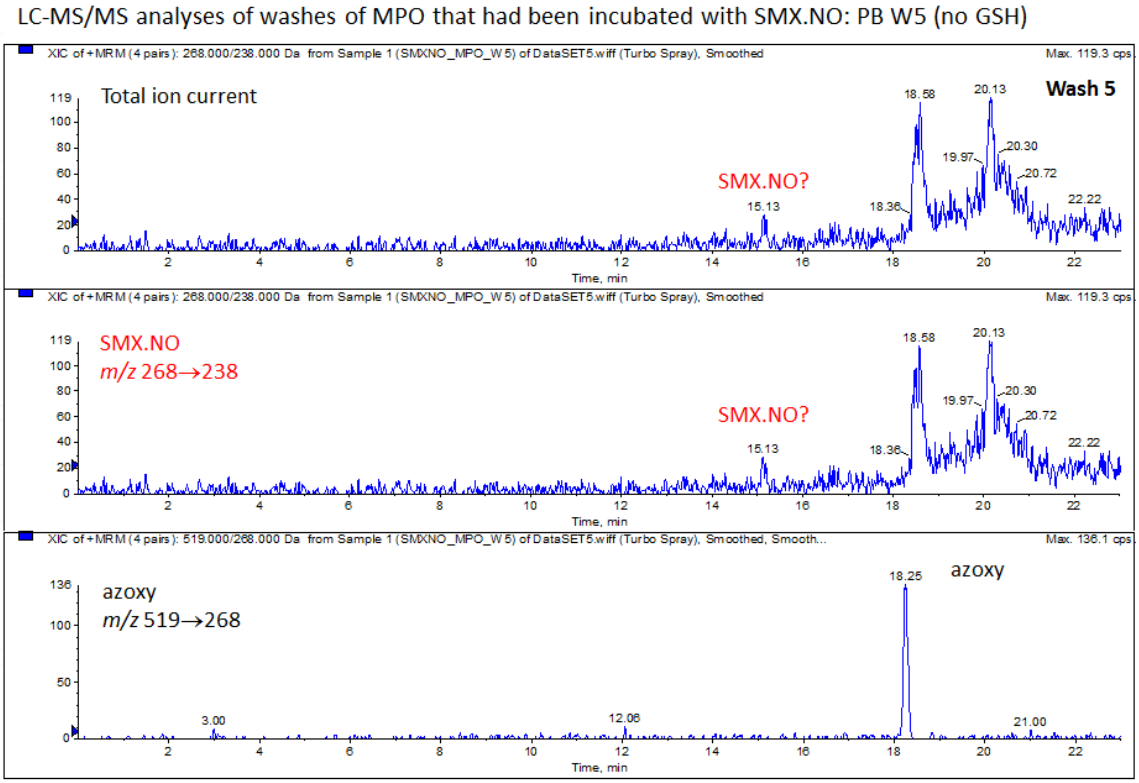


Figure 6.21 LC-MS/MS analysis of 5th wash (PB W5) of SMX.NO-modified MPO with no convincing evidence of SMX.NO signal.

The phosphate buffer+GSH washes (PB+GSH W1-W5) of filtered MPO that had been incubated with SMX.NO were analysed by LC-MS/MS immediately after they were thawed from -20°C. These GSH-containing washes and the PBS washes analysed separately were sampled in reverse order, i.e. starting with W5, to avoid/minimize sample contamination by 'carry over'. The first wash clearly contained a high concentration of SMX.NO and also contained SMX.NHOH. The second wash, oddly, did not contain any of the analytes. No explanation for this observation is evident. The 2nd PBS wash contained abundant amounts of SMX.NO and azoxy (figure 6.19), and even the 3rd PBS wash contained an appreciable quantity of SMX.NO. The 3rd and 4th PBS+GSH

washes contained substantial amounts of hydroxylamine but no nitroso was detected (figure 6.27, appendix IV). Apparently the remaining SMX.NO associated with the filtered MPO underwent quantitative reduction to SMX.NHOH by GSH between the first and third washes (Cribb et al. 1991); and the SMX.NHOH was eluted by the third and fourth washes. The fifth PBS+GSH wash contained no more than a trace of SMX.NHOH and no detectable SMX.NO (figure 6.22). The azoxy dimer was the only confidently identifiable compound in this wash.

Following successful modification with SMX.NO and characterization of the MPO adduct, quantification of the nitroso-protein adduct was performed using Bradford assay. The recovery of unmodified and SMX.NO-modified MPO was 74% and 77% respectively.

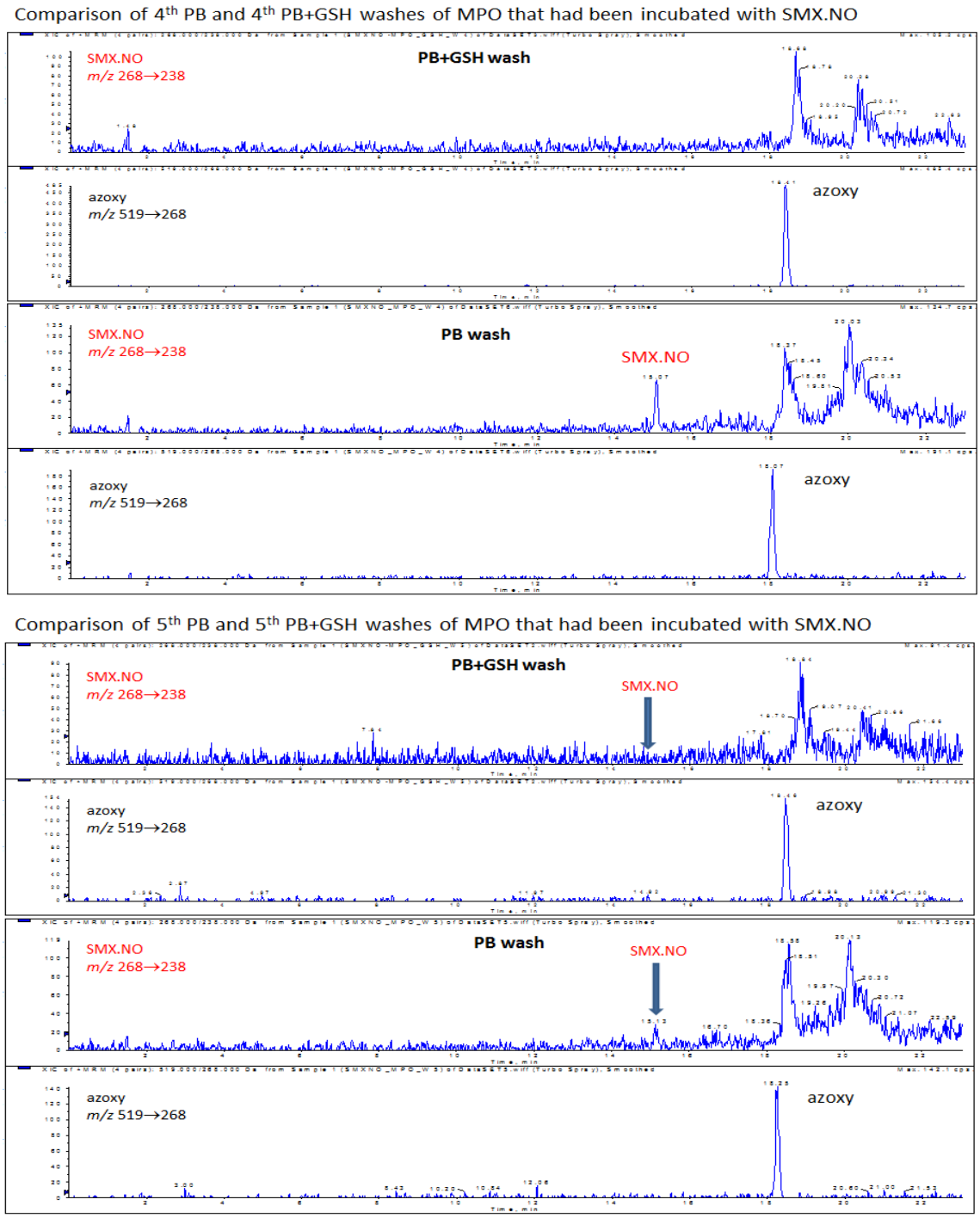
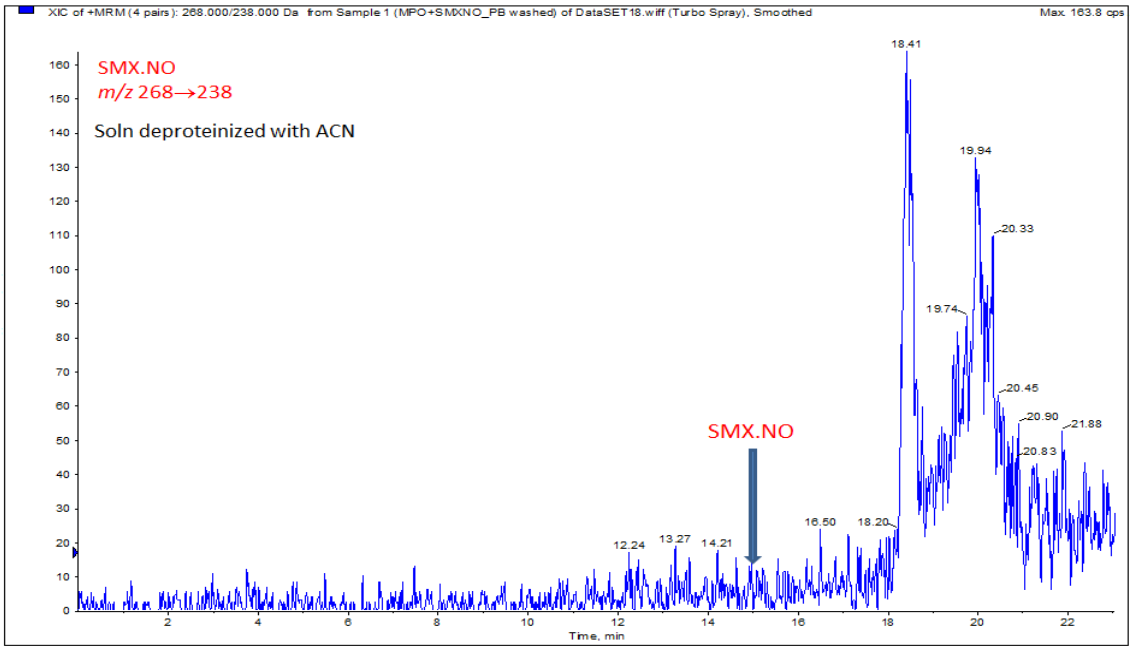


Figure 6.22 Comparative LC-MS/MS analysis of filtrate from PBS and PBS+GSH washes for the 4th and 5th washes. A trace of SMX.NO was detectable in the 4th PB wash but absent in the 5th PBS wash. SMX.NO was totally absent from both washes.

LC-MS/MS analysis of solution of PB-washed MPO that had been incubated with SMX.NO



LC-MS/MS analysis of solution of PB+GSH-washed MPO that had been incubated with SMX.NO

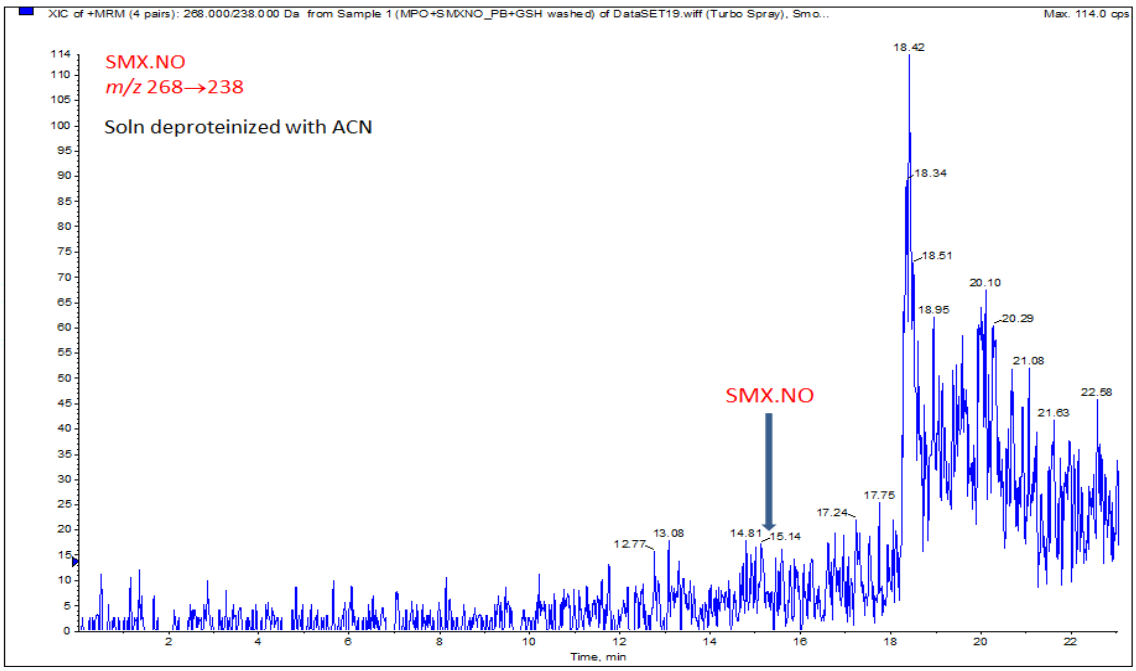
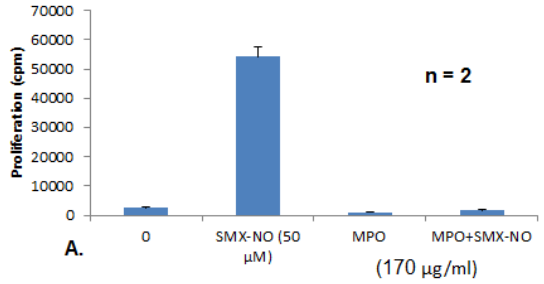
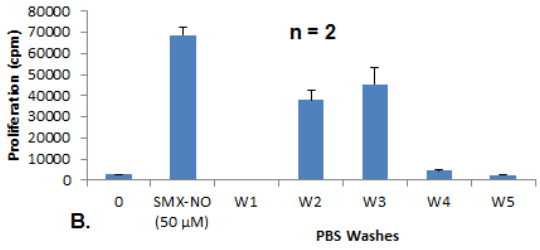


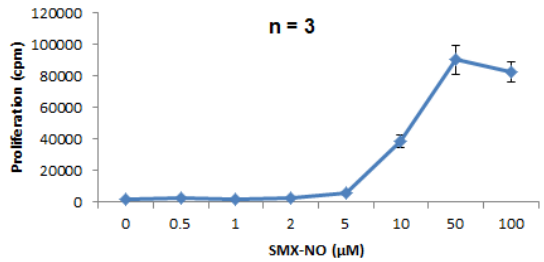
Figure 6.23 LC-MS/MS analyses of supernatants of solutions of unmodified MPO after 5 times PBS or PBS+GSH. The MRM chromatograms suggest an absence SMX.NO from both protein samples. The solutions of the washed MPO were deproteinated with ACN (5 µL + 75 µL ACN)

6.4.2 Determination of immunogenicity of SMX.NO-modified MPO

The immunogenicity of SMX.NO-modified MPO adducts was determined using a 48-hour proliferation assay. Also, the T-lymphocyte response to fractions (100 µL) of ultrafiltrate was investigated using the same TCCs. Data obtained suggested that the SMX.NO-modified MPO adduct did not induce a T-cell response (figure 6.24A). The SMX.NO concentration in the fraction obtained from the first PBS wash was estimated by LC-MS/MS using peak height of SMX.NO standard. Ultrafiltrate from the first wash contained 1,400 µM SMX.NO and induced cytotoxicity (Figure 6.24B and D). According to Naisbitt et al. (1999), human lymphocytes are sensitive to the cytotoxic effects of SMX.NO when the nitroso-SMX concentration is 1,000 µM. Approximately 46.8±2.8% of T-lymphocytes were unable to exclude trypan blue after a 1 hour incubation (Naisbitt et al. 1999). T-lymphocyte proliferation following incubation with ultrafiltrates from the 2nd and the 3rd PBS washes was consistent with the presence of soluble SMX.NO, approximately 170 µM and 19 µM, respectively. Hence, filtrates from washes 2 and 3 activated TCCs while other washes did not. Also, TCCs were activated with SMX.NO and not the modified peptide (figure 6.24B). Furthermore, the SMX.NO concentration-response relationship of drug-specific TCCs suggested a significant T-lymphocyte proliferation between 5-10 µM (figure 6.24C).







PBS Wash	Theoretical residual [SMX-NO] (µM)	LC-MS/MS analysis of [SMX-NO] (µM)
W1	1915.3	1400
W2	112.66	170
W3	6.63	19
W4	0.38	-
W5	0.02	-

D.

Figures 6.24 (A) [^{3}H]-thymidine proliferation assay to determine immunogenicity of SMX.NO-modified MPO adduct. SMX.NO-specific T-cell clones (5×10^4 cells, 50 µL) were co-cultured with irradiated autologous EBV-transformed B-cells (1×10^4 cells, 50 µL) in the presence of either unmodified MPO or SMX.NO-modified MPO (0.17 mg/mL, 100 µL). SMX.NO (50 µM, 100 µL) and culture medium (100 µL) were used as positive and negative controls, respectively. The assay was performed in a 96-well U-bottom microplate. The plate was incubated at 37°C, under an atmosphere of 95%O_2/ 5% CO_2, for 48 h. [^{3}H]-thymidine (0.5 µCi) was added for the final 16 h of incubation and T-cell proliferation evaluated using scintillation counter. **(B)** SMX.NO- specific T-lymphocyte proliferation in response to fractions of PBS washes of modified MPO (W1-W5). Cells were co-cultured with antigens as described above. **(C)** Estimation of the minimum concentration of SMX.NO to induce drug-specific T-lymphocyte proliferation. **(D)** Calculated (based on dilution) and instrumentally estimated (LC-MS/MS) residual SMX.NO concentrations after each PBS wash.

6.5 Discussion

Biotransformation is a critical aspect of the pharmacology and the elimination of most drugs. Certain chemically inert drugs undergo biotransformation to generate highly protein-reactive intermediates capable of 'provoking' the immune system and resulting in adverse drug reactions, usually with cutaneous manifestations (Roychowdhury and Svensson 2005). Although the liver is the major organ involved in drug metabolism, the stability of these metabolites in extracellular transit or in the systemic circulation is questionable (Uetrecht 1992; Reilly et al. 2000). Therefore, extra-hepatic metabolism involved in localised generation of protein reactive intermediates might be more relevant for the activation of immune cells (Uetrecht 1992; Vyas et al. 2006; Roychowdhury et al. 2007; Sharma and Uetrecht 2013). In this respect, myeloperoxidase might be important in the metabolism of SMX (see chapters 1 and 2). The protein reactive SMX.NO is thought to be responsible for the high incidence of immune-mediated hypersensitivity to SMX (Vilar et al. 2003). Although SMX.NO adduction of HSA has been well defined *in vitro* and cysteine-34 shown to be the amino acid modified (Callan et al. 2009), SMX.NO's ability to modify other cysteine-containing proteins has not been researched.

According to Lavergne et al. (2008), anti-neutrophil cytoplasmic antibodies are associated with vasculitis in humans. Furthermore, sulphonamide induced hypersensitivity reaction presents with clinical signs suggestive of vasculitis (Lavergne et al. 2008). The authors also reported a significantly higher level (50%) of anti-MPO antibodies in sulphonamide-hypersensitive dogs than in sulphonamide tolerant dogs. Collectively, their data suggest that SMX-modified MPO is involved in the drug-specific humoral response. Thus, the studies

described in this chapter investigated the *in vitro* SMX.NO modification of recombinant myeloperoxidase and MPO-derived peptides, and assessed the immunogenicity of SMX.NO-modified MPO and MPO-derived peptides. SMX.NO haptenation of recombinant MPO generated the N-hydroxysulphinamide adduct (Figure 6.1) on Cys309 and Cys398 but not on Cys316 or Cys319. The tertiary structure of a protein will determine the pre-reaction (non-covalent) binding of a small molecule electrophile, which will influence the ultimate site(s) of covalent adduction (Skipper 1996). Furthermore, the reactivity (nucleophilicity/pK_a) of amino acid side chains can be influenced considerably by the protein's conformational structure (Skipper 1996; Jenkins et al. 2009). The pre-reaction binding can have a decisive influence on which moderately or highly reactive side chains are adducted by a particular electrophilic compound or metabolite (Fry et al. 1998). Modifying the full-length recombinant MPO provided experimental quantities of SMX.NO-modified tryptic peptides.

SMX.NO-modified MPO was purified using ultrafiltration. Washes were performed with either plain PBS or PBS containing GSH. LC-MS/MS was used to confirm the removal of free SMX.NO. However, an assessment of T-cell proliferation revealed that SMX.NO-modified MPO failed to activate drug-specific TCCs. Western blotting indicated that SMX.NO bound irreversibly to MPO. LC-MS/MS analyses revealed marked differences between the modifications of MPO-derived synthetic peptides MPO1 and MPO3 and the full-length recombinant MPO. The reaction of SMX.NO with MPO1 yielded N-hydroxysulphinamide adducts on Cys309 and 319 (mass increment of 283 amu). Incubation of SMX.NO with MPO3 generated a peptide with two SMX.NO-derived modifications, namely a sulphinamide (mass increment of 267 amu)

and an N-hydroxysulphinamide adduct on Cys316. Because a sulphinamide adduct is formed by the reaction of a nitroso with a thiol (Callan et al. 2009), this observation demonstrates that at least the side chain of Cys316, if only in MPO3, can persist fractionally in the unoxidized state *in vitro*. All of the other haptenated MPO cysteines were detected as N-hydroxysulphinamide adducts, and therefore by implication were formed from sulphenic acid derivatives (Figure 6.1).

Although SMX.NO readily modified the primary synthetic peptide, MPO1 (^{307}ADCIPFFRSCPACPG^{321}), the formation of both intra- and inter-chain disulphide bonds was a major analytical limitation, and affected the degree of modification and the chromatographic peptide purification adversely. The mass spectra generated from these cysteine-bridged peptides were difficult to interpret precisely. Due to the presence of multiple proline residues, the peptide tended to fragment internally rather than giving readily interpretable b- and y-ion series (Bleiholder et al. 2011). Alanine substitution of all the cysteine residues present in MPO1, which produced MPO2, was expected to abolish SMX.NO modification. LC/MS/MS analysis of the peptide recovered from incubations of MPO2 and SMX.NO showed there was no MPO2 haptenation. This result proves the critical role cysteine residues play in SMX.NO-protein haptenation (Callan et al. 2009). MPO3 (one cysteine residue) haptenated with SMX.NO generated spectra that were much easier to interpret than those generated from haptenated MPO1 (three cysteine residues).

An attempt was made to both remove unbound SMX.NO and purify the modified peptide (MPO1) by off-line reversed phase (RP) chromatography. To

circumvent the formation of disulphide bonds in MPO1 and enhance SMX.NO modification, another MPO-derived (MPO3) peptide was synthesised. MPO3 (^{307}ADAIPFFRSCPAAPG^{321}) had just one cysteine residue, at position 316. Interestingly, analyses of the MPO1 adducts revealed two SMX.NO-modified peptides, with 283 mass additions on Cys309 and Cys319, but MPO1 was not detected as a sulphenic acid derivative, which is the putative precursor of an N-hydroxysulphinamide adduct (Figure 6.1).

Glycosylation of the Asn323 in MPO *might* hinder access of SMX.NO to Cys316 (see figure 6.16). MPO1 (^{307}ADCIPFFRSCPACPG^{321}) is a product of chemical synthesis and does not contain *any* glycosylated amino acid. Steric hindrance of the reaction of a small molecule electrophile with an amino acid side chain is not a plausible limitation to interaction in the case of short peptides; which cannot assume the complex tertiary structures that set up multi-point interactions between small molecules and proteins (Skipper 1996; Fry et al. 1998).

Half of human MPO consists of two polypeptides of 108 and 466 amino acid residues, respectively, including six potential sites of asparagine-linked glycosylation, namely Asn139 (Liu et al. 2005), Asn323 (Ramachandran et al. 2006; Van Antwerpen et al. 2010), Asn355 (Chen et al. 2009; Van Antwerpen et al. 2010), Asn391 (Baron et al. 2001; Van Antwerpen et al. 2010), Asn483 (Liu et al. 2005; Chen et al. 2009; Van Antwerpen et al. 2010), and Asn729 (Van Antwerpen et al. 2010). All the potential sites for asparagine-linked glycosylation reside in the larger polypeptide (Johnson et al. 1987; Morishita et

al. 1987). Cys309 is evidently accessible to nitroso-SMX and is the cysteine residue modified by SMX.NO in MPO1.

Alternatively, the oxidation and disulphide status of the cysteine residues in recombinant MPO may not be the same as those in the native protein (Zeng and Fenna 1992), leading to alternative favoured sites of modification. Redox states and disulphide bridging status of recombinant proteins may differ from those of native proteins, with possible biological and functional implications (Monie et al. 2005). Furthermore, arylnitroso-protein cysteinyl adducts can be unstable (Liu et al. 2008); hence, a SMX.NO-Cys316 adduct might have degraded during processing of protein and/or peptides for LC-MS/MS analysis. In particular, enzymatic digestion of a modified protein containing an arylnitroso-derived sulphinamide adduct can, in some cases, cause complete hydrolysis of the adduct, producing an essentially stable cysteinyl sulphinic acid (Wang et al. 2005; Liu et al. 2008).

Although purification of SMX.NO-modified MPO3 was carried out using reversed phase HPLC and cation exchange chromatography to exclude residual SMX.NO, a low adduct recovery (1.9 µg/60 µL) was observed. Concentrations of MPO3 adducts used in the proliferation assay ranged from 0.03-0.3 µM. To circumvent the low concentration of recovered SMX.NO-modified MPO3, full-length recombinant MPO was incubated with SMX.NO. Purification and concentration of the resulting adduct was performed using an ultrafiltration technique involving either PBS or PBS+GSH washes to remove free SMX-NO. Analyses of PBS+GSH washes are presented in appendix iii-v. A comparison of the 4th and 5th 'PBS' and 'PBS+GSH' washes suggested the 'PBS+GSH' wash method was more

effective at removing SMX.NO from filtered MPO that had been incubated with SMX.NO (figure 6.22). The reduction of SMX.NO to hydroxylamine observed during the PBS+GSH washes is consistent with previously published data (Cribb et al. 1991; Burkhart et al. 2001; Sanderson et al. 2007). With the unequivocal elution of SMX.NO from MPO adduct in the 5th PBS and 5th PBS+GSH washes, further LS-MS/MS analysis on the washed MPO adduct was performed to confirm the removal of SMX.NO. A single solution of the MPO that had been washed five times with either PBS or PBS containing GSH, after incubation with nitroso, was deproteinized with ACN (5 µL + 95 µL ACN or 75 µL ACN, respectively). The supernatants were analyzed immediately. No signal corresponding to SMX.NO was detected in either supernatant (figure 6.23). No azoxy was detected in either supernatant. The azoxy dimer is incapable of stimulating T-cells (Naisbitt et al. 2002). Allowing for the 15–fold dilution of the PBS+GSH solution of washed MPO, which was required for deproteinization and sample recovery, any residual SMX.NO in this solution would have been detected if its concentration had been ≥ 30 µM (≡ 1.9 µM in the deproteinized supernatants). This was estimated from LC-MS/MS analysis of authentic SMX.NO.

Despite successful protein modification, characterisation, purification and definition of the chemistry involved in the haptenation of MPO by SMX.NO, T-lymphocyte proliferation data to determine immunogenicity was negative. The chemistry and immunogenicity of MPO haptenated by SMX in the presence of hydrogen peroxide was not explored due to time constraint. It will be interesting to compare MPO modifications by either SMX or SMX-NO. Hence, further experiments would be required to define the functional significance of

the observed *in vitro* MPO modification. Finally, future work would investigate such modifications *in vivo* and their involvement in the pathophysiology of SMX hypersensitivity reactions.

Chapter 7: Final discussion

Adverse drug reactions are common and can occur with some drugs. The clinical symptoms of ADRs are drug dependent and range from mild reactions like nausea to severe (sometimes life-threatening) side effects like anaphylaxis. ADRs have impact on patients, clinicians and the pharmaceutical industry. Drug hypersensitivity limits therapeutic options for treating diseases but also results in high morbidity and can sometimes be fatal. In the United Kingdom, the financial burden of ADRs to the NHS was estimated to be over £466 million annually (Pirmohamed et al. 2004). It takes an average of 10 years to develop a new drug and costs approximately $866 million (Adams and Brantner 2006). Therefore, the cost of withdrawing a molecular entity due to serious ADRs is enormous; hence, the need to predict these reactions at the early stages of research and development.

Although about 80% of hospital admissions resulting from ADRs are linked to type A reactions, most of the mortalities occur from type B hypersensitivity reactions (Routledge et al. 2004). The rise in the number of drug hypersensitivity reactions linked to HLA suggests that these reactions are immune-mediated. Recent advances characterizing the nature of the drug-specific immune response in susceptible patients' means that the disease pathogenesis is now better understood and hopefully with further research may eventually be managed (Torres et al. 2003; Nassif et al. 2004; Beeler et al. 2006; Blanca et al. 2009).

SMX is used in combination with trimethoprim to treat opportunistic infections in HIV/AIDS patients but also in the management of recurrent respiratory tract

infections in patients with CF. The main clinical symptom of SMX hypersensitivity is skin rashes of varying severity (Schnyder et al. 2000). These reactions are thought to be mediated by SMX (antigen)-specific T-lymphocytes (Maurihellweg et al. 1995; Schnyder et al. 1998). Although the high frequency of SMX-hypersensitivity in susceptible patient populations has been long established (Bayard et al. 1992; Pirmohamed and Park 2001; Naisbitt 2004), the reasons for the increased number of reactions has not been defined. The majority of SMX undergo *N*-acetylation, a reaction catalysed by N-acetyltransferases enzymes, to form nontoxic metabolites that are safely excreted (Cribb et al. 1993; Schnyder et al. 2000). Hepatic oxidation of SMX by CYP2C9 and MPO generates SMX.NHOH, which is further oxidized to form the protein-reactive metabolite, SMX.NO, which is implicated in SMX hypersensitivity (Park et al. 1987; Cribb et al. 1990; Cribb and Spielberg 1992; van der Ven et al. 1994; Vyas et al. 2006). In the absence of an underlying disease, it is thought that all the reactive metabolites generated in the liver will be detoxified by glutathione conjugation. Therefore, it is unlikely that the small amounts of SMX.NO that escapes this detoxification pathway will migrate to the skin, modify protein and generate adducts that are ultimately processed by antigen presenting cells to liberate peptide antigens that activate T-cells. Hence, we propose an alternative hypothesis; specifically, immune cells that reside in the skin generate SMX.NO locally. Covalent modification of skin protein will subsequently generate neoantigens that drive SMX induced cutaneous reactions.

SMX represents an ideal drug for the study of hypersensitivity because its metabolism has been defined and patient samples are available for functional

studies. Furthermore, stable and reactive metabolites have been synthesised and available for research (Naisbitt et al. 1996). Finally, SMX-specific, SMX.NO-specific and cross-reactive TCCs have been generated to study immunological and pathophysiological mechanisms of SMX hypersensitivity (Schnyder et al. 1997; Schnyder et al. 2000; Elsheikh et al. 2011).

The aim of this research project was to characterize the metabolic and immunological factors that are responsible for SMX hypersensitivity. A total of 1336 TCCs were generated and tested for drug specificity. TCCs were generated from 5 SMX-hypersensitive patients and 1 SMX-naïve volunteer. Sixty-eight TCC were SMX.NO-specific and expressed the CD4 cell surface protein. Only 1 TCC responded to SMX, and expressed the CD8 cell surface protein. No cross-reactivity observed with the SMX.NO-specific TCCs. Drug-specific TCCs secreted T_H1 and T_H2 cytokines but also cytotoxic molecules like granzyme-B, perforin and Fas ligand. In another study, most of the TCCs generated from SMX-hypersensitive patient with cystic fibrosis were SMX.NO-specific and showed cross reactivity with SMX (Elsheikh et al. 2010). The differences observed in the specificity of TCCs generated may be due to the patients involved.

The following aspects were examined (1) the expression and functionality of MPO in immune cells and the immunogenicity of SMX.NO-modified MPO-derived peptides; (2) the involvement of specific HLA molecules in the presentation of SMX.NO-derived antigens to drug-specific TCCs; (3) the role of co-inhibitory receptor-ligand interactions (especially the PD-1/PD-L signalling pathway) in regulating the priming of naïve T-cells to SMX.NO; (4) the

cytokines/cytolytic molecules secreted by antigen-specific TCC; and (5) the priming of naïve and memory T-cells to SMX and SMX.NO.

To determine whether immune cells resident in the skin are capable of SMX metabolism and therefore generating SMX.NO-modified peptides that drive cutaneous reactions, we investigated the enzyme expression profile and the SMX metabolism in EBV-transformed B-cells, DCs and HL60 cells. EBV-transformed B-cells were used as they are the cell type most frequently used as antigen presenting cells in TCC assays. DCs are professional antigen presenting cells utilised in the T-cell priming assay discussed in detail (chapters 4 and 5). HL60 cells are a neutrophil cell lines used as a positive control as they have previously been shown to express high levels of MPO (Hope et al. 2000; Wagner et al. 2001). Using an anti-drug antibody in an ELISA experiment, SMX.NO-protein adducts was detected in DCs, HL60s and EBV-transformed B-cells. This suggested that each cell type was metabolically active and able to metabolise SMX to its protein-reactive metabolite, SMX-NO. Western bolting analysis revealed low MPO expression in EBV-transformed B-cells, when compared with DCs and HL60 cells, consistent with the ELISA data. Mass spectrometry was then used to identify MPO in the different immune cells. MPO was clearly detectable in HL60 cells; however, it was difficult to obtain conclusive data in EBV-transformed B-cells. Despite this, RT-PCR confirmed mRNA for MPO in EBV-transformed B-cells.

A significantly higher level (50%) of anti-MPO antibodies has been reported in sulphonamide-hypersensitive dogs than in sulphonamide tolerant dogs thus suggesting SMX-modified MPO is involved in the drug-specific humoral

response (Lavergne et al. 2008). Hence, the immunogenicity of SMX.NO-modified MPO-derived peptides was investigated. SMX.NO-modified MPO adducts were successfully generated *in-vitro* and characterised using LC-MS/MS analysis (chapter 6). Modification of the full length recombinant MPO revealed the formation of N-hydroxysulfinamide adduct on Cys309 and 398. However, LC-MS/MS analyses of SMX.NO-modified MPO peptides were complicated by different levels of intra-and inter-peptide disulphide bond formation. Three HLA-DQ binding peptides, the HLA molecule involved in the activation of SMX.NO-responsive T-cells (see below), based on the structure of MPO and incorporating Cys309 were then synthesized for functional studies. MPO1 (^{307}ADCIPFFRSCPACPG^{321}) was a native peptide containing Cys309 and a 2 extra Cys residues at positions 316 and 319. MPO3 (^{307}ADAIPFFRSCPAAPG^{321}) was essentially the same as MPO1 with Cys 309 replaced with an alanine group. In MPO2 all 3 Cys residues were replaced with alanine; hence, this peptide was to serve as a negative control. Mass spectrometric analysis of SMX.NO modified MPO1 and MPO3 revealed that the drug metabolite bound to the Cys groups in a number of ways. The *N*-hydroxysulfinamide moiety (283 amu) was detected on Cys309 in MPO1 while the sulphinamide and N-hydroxysulfinamide were detected in MPO3 at the same position. No modification was observed on Cys 316. Both intra-and inter-disulphide bonds were detected in MPO1 and MPO3, and complicated the interpretations of spectra obtained. As expected, SMX.NO modification of MPO2 (^{307}ADAIPFFRSAPAAPG^{321}) was not observed. Despite convincing data showing MPO modification for MPO1, MPO3 and recombinant full length MPO, SMX.NO-specific TCCs failed to proliferate in response to MPO adducts. It is unclear at this point whether the modifications observed with

MPO are irrelevant to the pathophysiology of SMX-induced cutaneous reactions. Further experiments are required to characterise MPO modification following incubation with SMX in the presence of hydrogen peroxide as a co-factor. Our studies focused on analysis of binding after the direct addition of SMX.NO. If the resulting modifications differed in terms of chemical structure or sites of modification, it then would be important to study T-cell immunogenicity. Our assays were also limited by two additional factors. First, the extensive extraction procedures that were performed to ensure that soluble SMX.NO was removed from MPO prior to analysis of T-cell responses severely restricted the quantity of adduct that could be added to the culture conditions. If the experiments were to be repeated, higher amounts of native protein should be used. Secondly, the T-cell clones used to detected responses to SMX.NO MPO adducts were generated thought the culture of PBMC with soluble SMX.NO. If these initial PBMC cultures contained the MPO adduct we may have been successful in detecting clones that were responsive towards the adduct, but not SMX.NO itself.

A customized PubMed database search of the terms "HLA" and "drug hypersensitivity" from 1994-2004 (http://www.ncbi.nlm.nih.gov/pubmed) revealed 137 results. A total of 579 results were obtained when the date is extended, 1994-2014. Most of the search results within the last decade describe new HLAs implicated in drug hypersensitivity and the successful application of these findings in clinics (Hetherington et al. 2002; Alfirevic et al. 2006; Kaniwa et al. 2008; Mallal et al. 2008; Daly et al. 2009; Bharadwaj et al. 2010; Kim et al. 2010; Chen et al. 2011; McCormack et al. 2011; Spraggs et al. 2011; Daly 2012; Han et al. 2012). However, the preferential drug presentation by the risk HLA

allele has only been defined and characterized for a limited number of drugs (abacavir, flucloxacillin, carbamazepine, allopurinol) (Chessman et al. 2008; Chen et al. 2011; Yun et al. 2012; Monshi et al. 2013; Lichtenfels et al. 2014; Yun et al. 2014). For many other forms of drug hypersensitivity HLA risk alleles have not been identified; however, it is still likely that the T-cell responses will be HLA-restricted in individual patients.

SMX hypersensitivity represents an ideal form of reaction that falls under this category. Independent studies from Liverpool, UK (Pirmohamed 2006; Alfirevic and Pirmohamed 2010) and France (Roujeau et al. 1986) have failed to detect HLA associations. Therefore, we examined the role of HLA molecules in the presentation of SMX.NO to drug specific-$CD4^+$ TCCs. Through antibody blocking experiments, the majority of TCCs generated from the SMX hypersensitive patients showed HLA-DQ-restricted SMX-NO-specific activation (chapter 3). APCs generated from SMX-naïve volunteers expressing a similar HLA-DQB1 allele as the patients' presented SMX.NO to drug-specific TCCs. Therefore, we propose that HLA-DQB1 plays a vital role in SMX.NO recognition by drug specific TCCs. Interestingly, the HLA-DQB1 allele involved in SMX.NO presentation differed in the individual subjects (figure 7.1). In contrast to HLA-DR and -DP, a range of amino acid residues with a diverse set of chemical specificities have been shown to interact with HLA-DQ molecules at key anchor positions. This has led to the suggestion that the whole peptide backbone contributes to MHC binding interactions. Somewhat surprisingly, approximately 25% of the peptide repertoire for an individual DQ molecule will overlap with other common DQ molecules. Based on these observations, we tested whether DQB1*05:01-restricted clones from patient 1 were also activated with SMX-NO

bound antigen presenting cells expressing DQB1*02:01. Interestingly, antigen presenting cells expressing DQB1*02:01 presented SMX.NO-derived antigen to TCCs generated from patient expressing DQB1*05:01. The cross-presentation may be because the different alleles expressed by both patients belong to the same serotype, DQ 2.5 (Sollid et al. 1989). These findings warrant further investigation in a larger patient cohort.

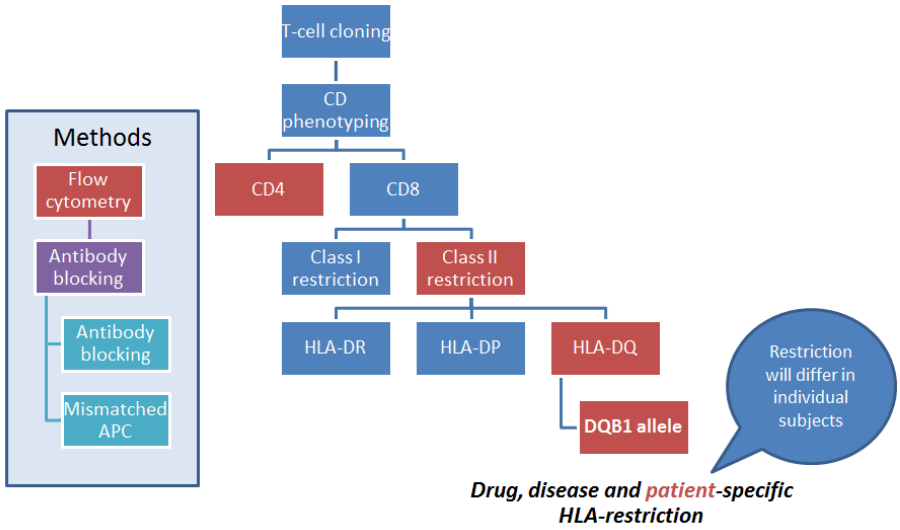


Figure 7.1-Schematic showing experiments performed and the observed HLA-DQ restriction in SMX.NO-specific $CD4^+$TCCs from SMX hypersensitive patients.

Although the antigenic signal presented on MHC molecules to T-cell receptors is vital for T-cell signalling, the overall outcome of a T-cell response is dependent on the activity of co-stimulatory and co-inhibitory receptors (Chen and Flies 2013; Liechtenstein et al. 2013). It is still not fully understood how the repertoire of co-signalling molecules downstream of the T-cell receptor regulate drug antigen-specific T-cell responses. Thus, this study investigated the

negative regulation by PD-L1 during drug-specific priming of IL-22 secreting T-cells and the influence of PD-1 on effector T-cells (chapter 5). The interaction of PD-1 expressed on surface of T-cells with its ligands (PD-L1/2) present on the surface of APCs result in cell cycle inhibition, inhibition of effector function, tolerance, exhaustion and apoptosis (Francisco et al. 2009; Wherry 2011; Fourcade et al. 2012; Chen and Flies 2013). T-cell priming involves the conversion of naïve T-cells from a dormant to an activated state regulated primarily by CD28 signalling (Shahinian et al. 1993). We investigated the effect of a PD-L1 blocking antibody on SMX.NO priming of naïve T-cells and the role of the PD-1/PD-L signalling on the effector response of SMX.NO specific TCCs. Using readouts for T-cell proliferation and cytokine secretion (IFN-ϒ and granzyme-B), we demonstrated enhanced priming of naïve T-cells from healthy donors to SMX.NO following blockade of PD-1/PD-L signalling (Gibson et al. 2014). Interestingly, blockade of this pathway had no effect on the proliferation of SMX.NO-specific $CD4^+$ TCCs although a modest increase in IFN-ϒ and granzyme-B was observed.

Priming of naive CD4+ and CD8+ T-cells against SMX.NO was found to be more effective when PD-L1 signalling was blocked. Upon restimulation, drug primed naïve T-cells proliferated more vigorously and secreted increased levels of IFN-γ, IL-13, and IL-22 but not IL-17. Although naive T-cells expressed low levels of PD-1, a transient increase in PD-1 expression was observed during drug-specific T cell priming. Drug-specific responses from *in vitro* primed TCCs from hypersensitive patients did not correlate with PD-1 expression. These findings suggest that the activation of naïve T-cells *in vivo* is regulated by numerous

cellular processes other than the drug antigen. Recently, targeted PD-1/PD-L1 therapies against many forms of cancers including melanomas have been reported to reactivate the immune system to fight cancer (Hong et al. 2014; Lu et al. 2014). Although no data exist at the moment, these therapies may deregulate drug-specific T-cells and increase the incidence of hypersensitivity to co-administered drug (s).

In terms of research applications, the DC-priming assay is currently being developed to explore T-cell responses to a variety of drug antigens, to explore HLA restriction and to determine the matrix of co-stimulatory/co-inhibitory receptor ligand interactions that regulate drug-specific T-cell priming. Currently, the assay cannot be used as a predictive drug allergy test during the pre-clinical stages of drug development; however, it might be useful in exploring whether second line drugs will be associated with similar immunological liabilities.

Most of the TCCs generated were $CD4^+$ and secreted IFN-γ, IL-5, IL-13, IL-22, granzyme-B and Fas ligand in response to drug stimulation. These findings suggest that $CD4^+$ cells may be implicated in the cutaneous phenotype of SMX hypersensitivity in these patients. Cytotoxic $CD4^+$ cells have previously been reported in a number of studies (Appay 2004; Hildemann et al. 2013). A more detailed analysis revealed two distinct cytokine profiles. TCCs secreted either FasL/IL-22 or granzyme B. The FasL/IL-22-secreting clones expressed the skin-homing receptors CCR4, CCR10, and CLA and migrated in response to CCL17/CCL27. IL-22 has previously been implicated in cutaneous skin reactions (Fujita et al. 2009; Miyagaki et al. 2011). We are currently isolating drug-specific

and bystander T-cells from inflamed skin of drug hypersensitive patients to characterize the role of different T-cell populations in the disease pathogenesis.

Although drug-specific T-cells have been extensively characterized in SMX hypersensitivity, it is still unclear why certain clones are SMX-responsive, while others are SMX.NO-responsive. Furthermore, the reason why some TCCs are cross reactive, responding to both SMX and SMX.NO via different mechanisms remains a subject of speculation. To investigate the molecular mechanisms that influence T-cell specificity to either SMX or SMX.NO, DC-priming of naïve and memory T-cell populations (from healthy donors) was performed followed by generation of drug-specific TCCs (chapter 4). A total of 240 TCCs were generated and evaluated for drug-specificity. Preliminary data suggests that while naïve T-cell population were primed to SMX.NO (n =9), memory T-cells were readily primed to SMX. On the other hand, drug-specific TCCs generated from PBMCs (pool of memory and naïve T-cells) were mostly SMX.NO specific. Experiments are ongoing to delineate the factors responsible for the observed selective priming of different populations of T-cells to either SMX or SMX.NO.

Put together, drug hypersensitivity is complex and influenced by a plethora of factors. Although different models have concentrated on a given aspect, only a holistic approach encompassing all the different aspects of research can provide a complete molecular template for understanding the pathogenesis of drug hypersensitivity. Continued research will translate into (1) the clinic through the development of tests that aid patient diagnosis and (2) Pharma through better drug design and synthesis.

Appendix

Appendix i

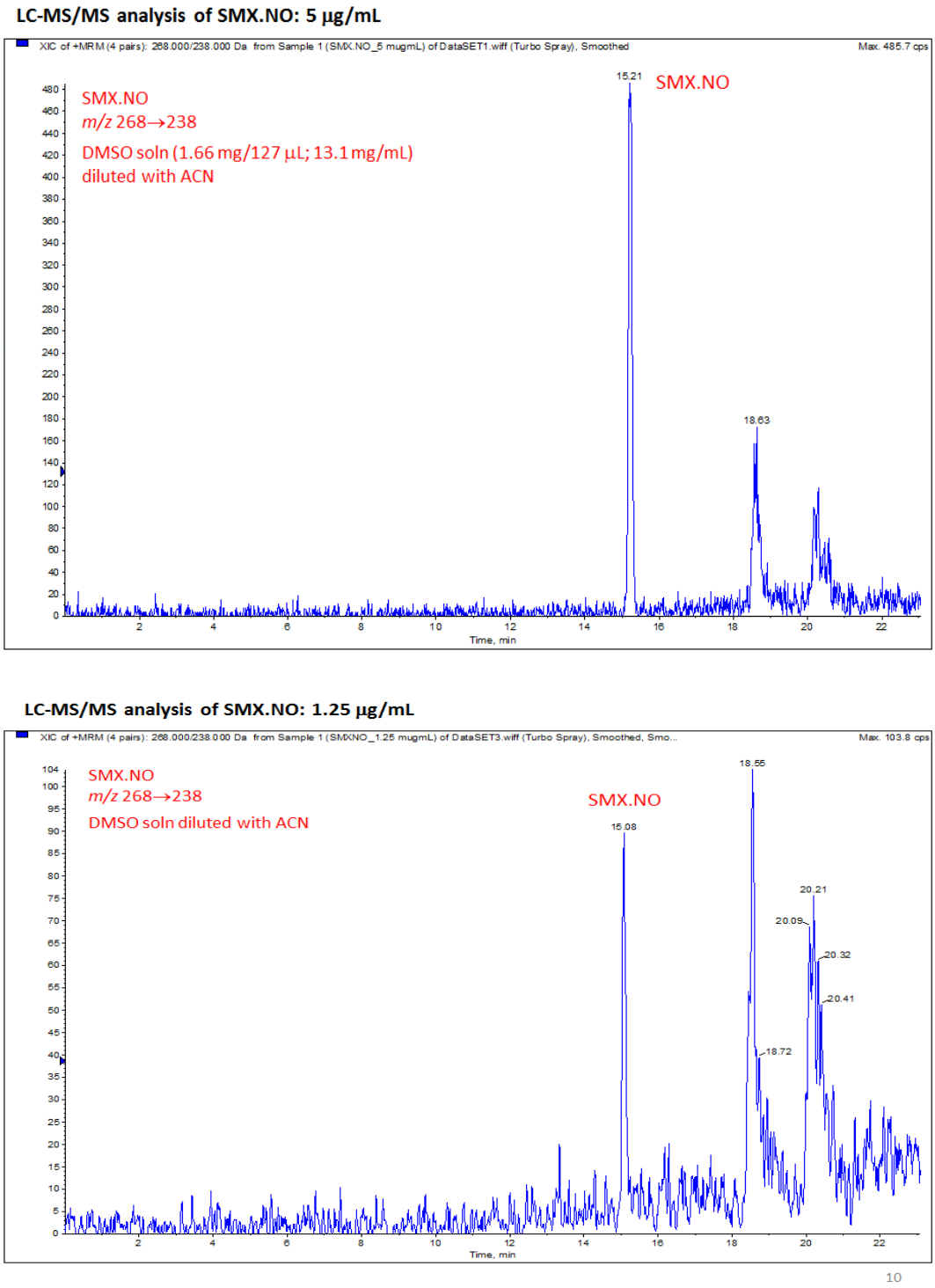


Figure 6.25 Limits of SMX.NO detection using LC-MS/MS. Distinctive peak corresponding to SMX.NO was obtained at 5 µg/mL (upper panel) and 1.25 µg/mL (lower panel).

Appendix ii

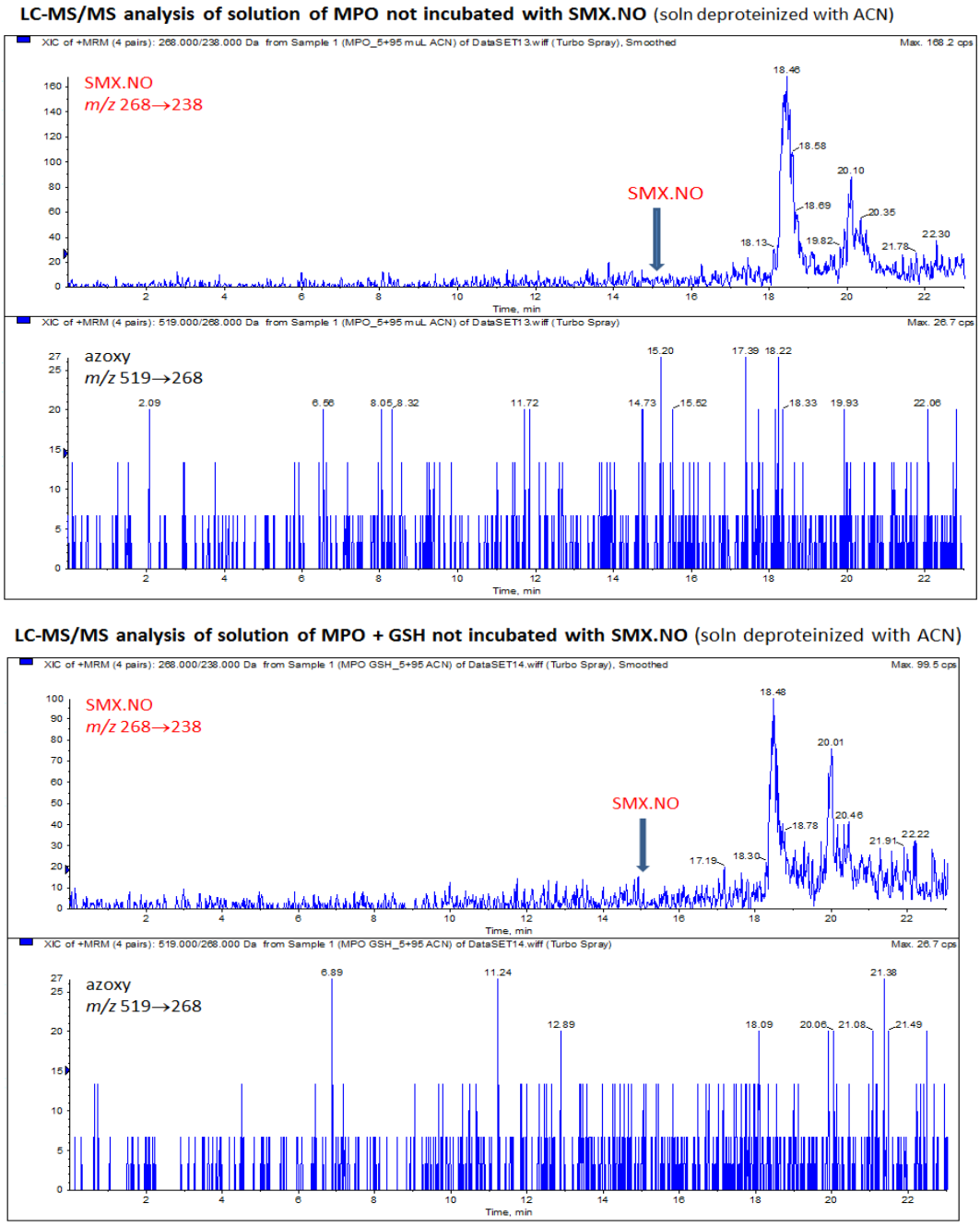


Figure 6.26 LC-MS/MS analysis for SMX.NO signal in control solution of MPO that had not been incubated with SMX.NO. As expected, no peak corresponding to SMX.NO was detected.

Appendix iii

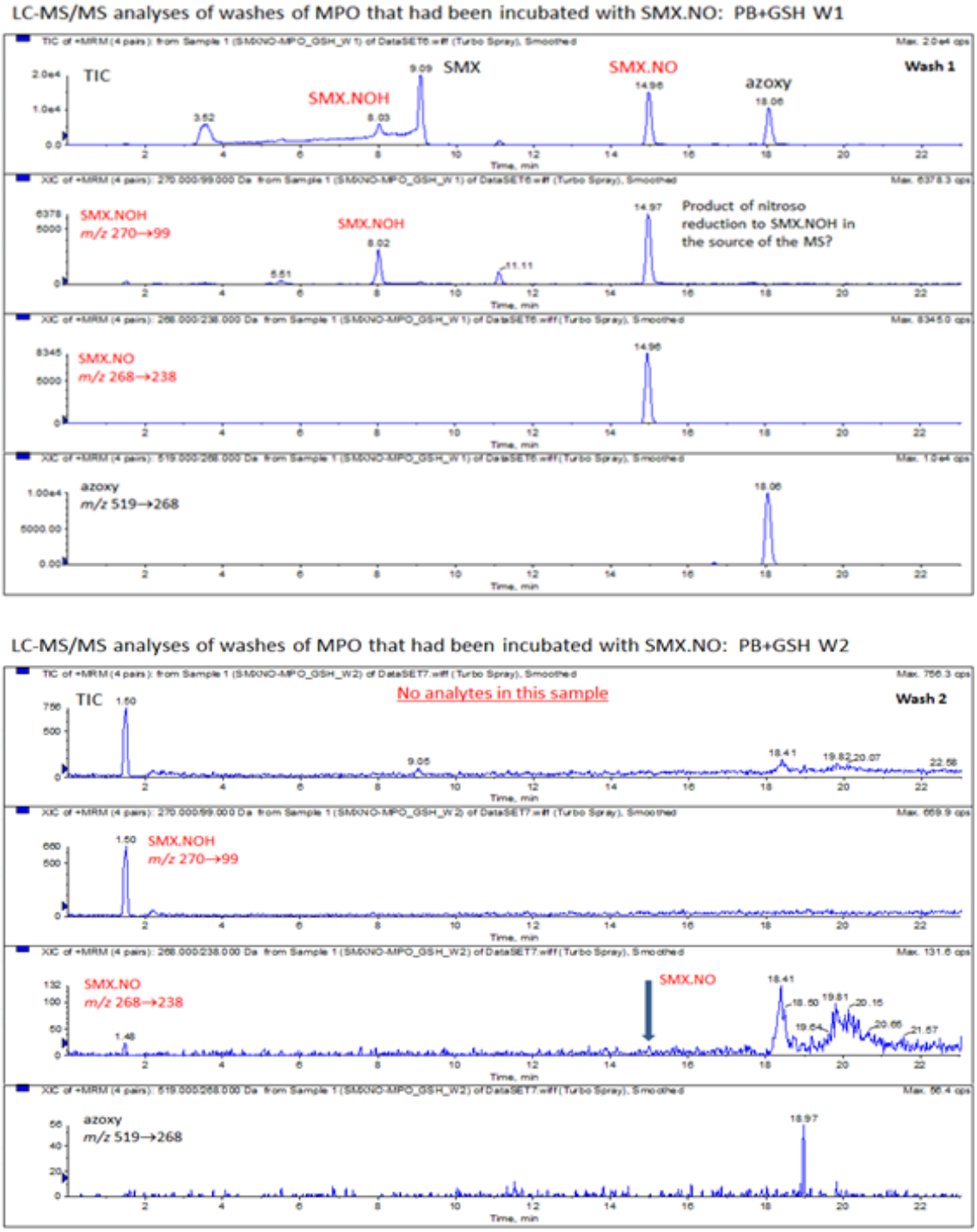


Figure 6.27 LC-MS/MS analysis of 1st and 2nd PBS+GSH washes (PBS +GSH W1 and W2) of SMX.NO-modified MPO adducts.

Appendix IV

LC-MS/MS analyses of washes of MPO that had been incubated with SMX.NO: PB+GSH W3

LC-MS/MS analyses of washes of MPO that had been incubated with SMX.NO: PB+GSH W4

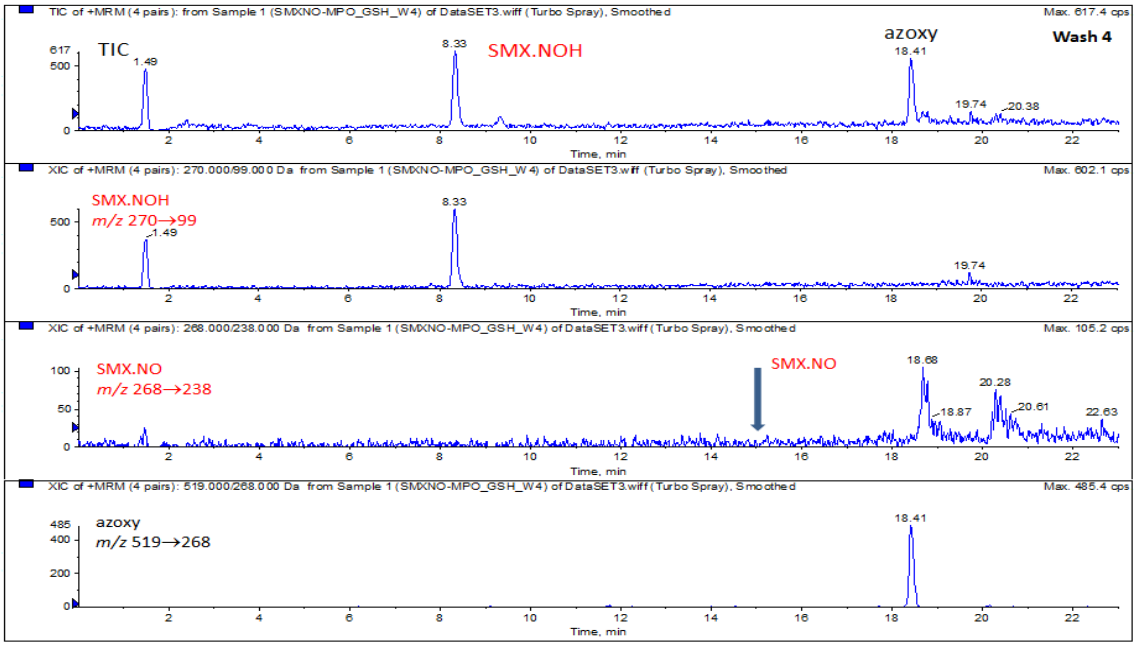


Figure 6.28 LC-MS/MS analysis of 3rd and 4th PBS+GSH washes (PBS +GSH W3 and W4) of SMX.NO-modified MPO adducts.

Appendix v

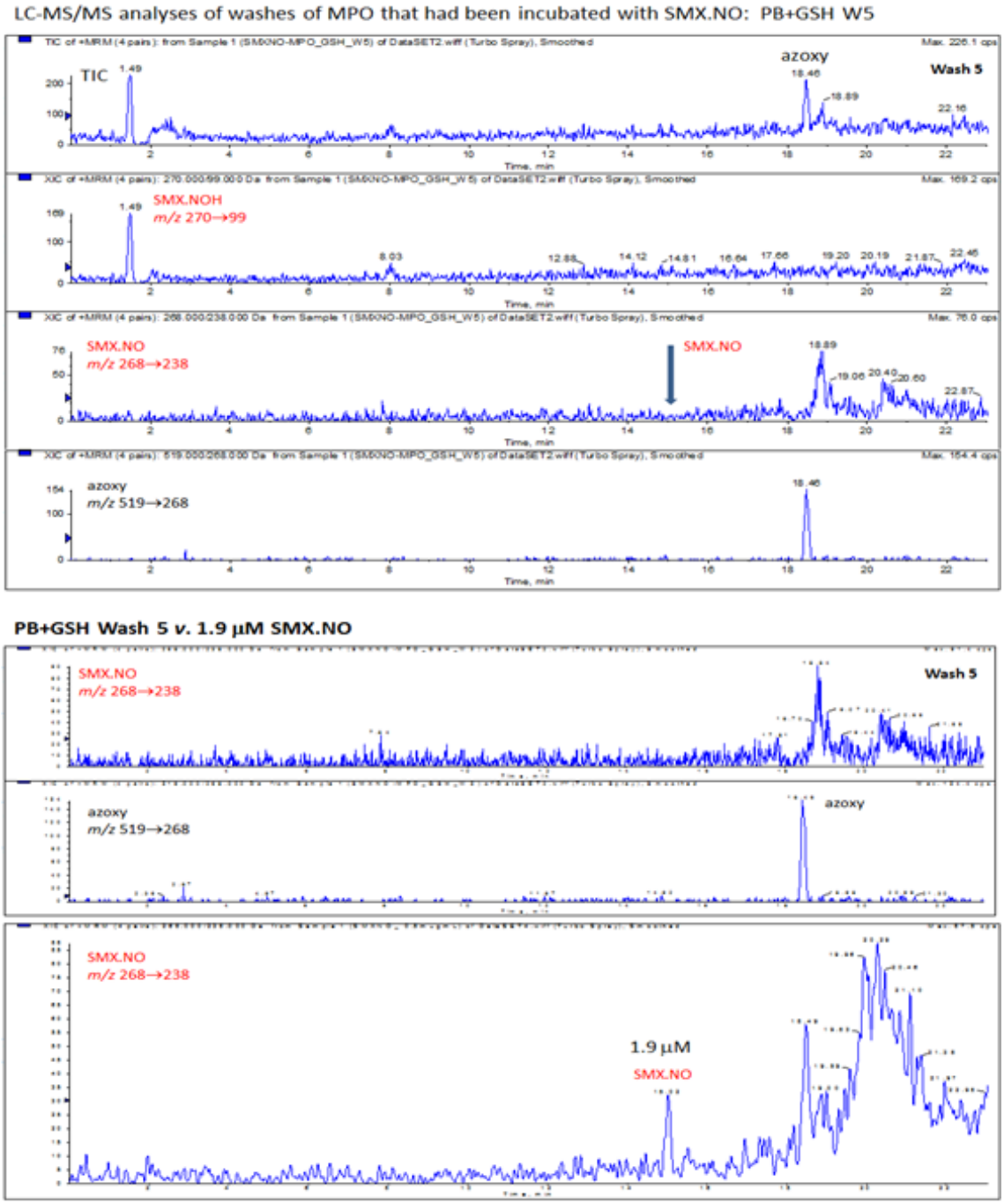


Figure 6.29 LC-MS/MS analysis of 5th PBS+GSH wash (top panel) and LC-MS/MS analysis of lowest detectable concentration of SMX.NO (lower panel).

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